AN INVESTIGATION INTO THE PHARMACOLOGICAL EFFECTS OF PHYTOCANNABINOIDS AND ENDOCANNABINOIDS IN HUMAN MESENTERIC ARTERIES

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ABSTRACT

Cannabinoids cause both acute and time-dependent vasodilation/vasorelaxation in a range of vascular beds. The vascular effect of cannabinoids is dependent on the cannabinoid ligand, the species studied and the vascular bed used. To date, there have been four studies that have characterised the effects of cannabinoids in humans. Therefore, the aim of this thesis was to characterise the pharmacological effects of cannabinoids in the human mesenteric artery.

Written informed consent was granted for the use of mesenteric arteries collected from patients at the Royal Derby Hospital. Arteries were dissected from mesenteric tissue and mounted on a Mulvany-Halpern myograph. Arteries were contracted using U46619 and endothelin-1. Concentration-response curves were carried out to the phytocannabinoids THC and CBD; the endocannabinoids AEA and 2-AG; the synthetic cannabinoids CP55,940 and HU-308. The underlying mechanisms of action were assessed using receptor antagonism, enzyme inhibition, endothelium denudation and ion channel manipulation. Experiments to probe the potential for cannabinoids to cause time-dependent vasorelaxation of human mesenteric arteries were also carried out. *Post-hoc* analysis was conducted on all acute vasorelaxation responses to assess the potential influence of patient characteristics/disease state on cannabinoid responses.

All cannabinoids tested, with the exception of HU-308, caused concentration-dependent vasorelaxation of human mesenteric arteries. The synthetic cannabinoid CP55,940 had the greatest R_{max} of all the cannabinoids tested. 2-AG had the greatest R_{max} of the endocannabinoids tested and CBD had the greatest R_{max} of the phytocannabinoids tested. Compared to animal models, cannabinoid efficacy was reduced in human mesenteric arteries. The vasorelaxant effects of 2-AG were mediated through COX-1 metabolism, prostanoid receptor activation (EP_4 and IP) and ion channel modulation. The mechanisms underpinning CBD-induced vasorelaxation were CB_1 and TRPV1 receptor activation, NO release, the endothelium and ion channel modulation. Vasorelaxant responses to AEA were inhibited by antagonism of the CB_1 receptor and a putative cannabinoid receptor located on the endothelium (CB_e), nitric oxide synthase inhibition and endothelium denudation. Cannabinoid responses were reduced in patients with cardiovascular diseases/disease risk factors including ischaemic heart disease, type-2 diabetes and hypercholesterolemia. Endocannabinoid responses were reduced in patients taking NSAID medication, with some reductions in responses seen to other medication including statins and beta-blockers. CBD and AEA were tested for time-dependent vasorelaxation. Both CBD and AEA were able to cause vasorelaxation that gradually increased over time, this was not mediated by the PPARγ receptor.

This thesis concludes that cannabinoids are able to modulate vascular tone in isolated human mesenteric arteries, and this may be blunted in patients with cardiovascular disease. Furthermore, this thesis presents data suggesting that differences exist between human and animal arterial responses to cannabinoids.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

Published review article

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Published abstracts and presentations

BPS Winter meeting, December 2010 (Poster presentation) Cannabinoid-induced vasorelaxation in the human mesenteric artery.
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C. P. Stanley, G. Manning & S. E. O'Sullivan

ICRS, July 2011 (Poster presentation) Cannabinoids improve endothelium-dependant vasorelaxation in Zucker diabetic fatty rat.
C.P. Stanley, A.J. Wheal, M.D. Randall & S.E O'Sullivan

ICRS, July 2011 (Oral presentation) - Characterisation of 2-arachidonoylglycerol-induced vasorelaxation in human mesenteric arteries.
C. P. Stanley, G. Manning & S. E. O'Sullivan

BPS Winter Meeting, December 2011 (Poster presentation) Cannabinoids improve endothelium-dependant vasorelaxation in Zucker diabetic fatty rat.
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BPS Winter Meeting, December 2011 (Oral presentation) Characterisation of cannabidiol-induced vasorelaxation in human mesenteric arteries
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www.pa2online.org/abstracts/vol9issue3abst098p.pdf

ICRS, July 2012 (Poster presentation) 2-AG responses are blunted in patients with cardiovascular disease and cardiovascular disease risk factors
C. P. Stanley & S. E. O'Sullivan

ICRS, July 2012 (Poster presentation) Cannabidiol induced vasorelaxation of human mesenteric arteries is mediated by the endothelium, CB_1 and TRPV1
C. P. Stanley & S. E. O'Sullivan

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ABBREVIATIONS

2-AG = 2-arachidonoylglycerol
Δ^9-THC = Δ^9-tetrahydrocannabinol
Δ^9-THC = Δ^9-tetrahydrocannabivarin
Aβ = β-amyloid
AA = Arachidonic acid
ABHD6 = α-β-hydrolase domain 6
ABHD12 = α-β-hydrolase domain 12
Abn-CBD = Abnormal cannabidiol
AC = Adenylate cyclase
ACh = Acetylcholine
AEA/Anandamide = *N*-arachidonoylethanolamine
AJA = Ajulemic acid
ARA-S = *N*-Arachidonoyl-L-serine
ATP = Adenosine triphosphate
BK = Bradykinin
BK_{ca} = Large conductance calcium sensitive potassium channel
cAMP = Cyclic adenosine monophosphate
Cannabinoids = collective term for phytocannabinoids, synthetic cannabinoids and endocannabinoids
CB_1 = Cannabinoid receptor one
CB_2 = Cannabinoid receptor two
CBD = Cannabidiol
CB_e = Putative endothelial cannabinoid receptor
CBG = Cannabigerol
CBGV = Cannabigerovarin
CBN = Cannabinol
CGRP = Calcitonin gene related peptide
CHO = Chinese hamster ovary
ChTX = Charybdotoxin
COX = Cyclooxygenase
COX-1 = Cyclooxygenase-1
COX-2 = Cyclooxygenase-2
CYP450 = cytochrome p450
DAG = Diacylglycerol
DAGL = Diacylglycerol Lipase
DETCA = Diethyldithiocarbamate
EDHF = Endothelium derived hyperpolarising factor
EET = Epoxyeicosatrienoic acid
ERK = Extracellular regulated kinase
FAAH = Fatty acid amide hydrolase
GIRK = G protein-coupled inward rectifying potassium channel
GP-NAEs = Glycerophospho-*N*-acylethanolamine
GPCRs = G protein-coupled receptors
GRP18 = G protein-coupled receptor 18
GPR55 = G protein-coupled receptor 55
GPR119 = G protein-coupled receptor 119
HEK = Human embryonic kidney cells
HETE = Hydroxyeicosatetraenoic acid
IbTX = Iberiotoxin
IK_{ca} = Intermediate conductance calcium sensitive potassium channel
iNAT = Ca^{2+} independent *N*-acyltransferase
K_{ATP} = ATP sensitive potassium channel
KPSS = High potassium physiological salt solution
L-NAME = N $^{(G)}$-nitro-L- arginine methyl ester
LBD = Ligand binding domain

LPI = L-α-lysophosphatidylinositol
lysoPI = Lysophosphatidylinositol
MβCD = Methyl-beta-cyclodextrin
MAFP = methylarachidonylfluorophosphonate
MAPK = Mitogen-activated protein kinase
MAGL = Monoacylglycerol lipase
MEK = Mitogen activated protein kinase kinase
mRNA = messenger ribonucleic acid
NAAA= *N*-acylethanolamine-hydrolysing acid amidase
NADA = *N*-arachidonoyldopamine
NArPE = *N*-arachidonoylphosphatidylethanolamine
NAPE-PLD = *N*-acylphosphatidylethanolamine phospholipase D
NEA = *N*-acylethanolamine
NF-κB = Nuclear Factor-κB
NFAT = Nuclear factor of activated T cells
NO = Nitric oxide
NOS = Nitric oxide synthase
OEA = *N*-Oleoylethanolamine
ODA = Oleamide
pAEA = Phospho-*N*-arachidonoylethanolamine
PEA = Palmitoylethanolamide
pEC_{50} = Inverse log of the concentration of drug needed to produce 50% maximal response
PG = Prostaglandin
PGD_2 = Prostaglandin D_2
PGE_2 = Prostaglandin E_2
$PGF_{2\alpha}$ = Prostaglandin $F_{2\alpha}$
PGH_2 = Prostaglandin H_2
Phytocannabinoid = Plant derived extracts of the *Cannabis sativa* plant
PI3K = Phosphoinositide 3-kinase
PIP2 = Phosphatidyl-inositol-bisphosphate
PKA = Protein kinase A
PKB = Protein kinase B
PLC =Phospholipase C
PPAR = Peroxisome proliferator-activated receptor
PPARα = Peroxisome proliferator-activated receptor alpha
PPARγ = Peroxisome proliferator-activated receptor gamma
PPARδ = Peroxisome proliferator-activated receptor delta
PPRE = PPAR response elements
PSS = Physiological salt solution
PTX = Pertussis toxin
RhoA = Ras homolog family member A
R_{MAX} = Estimate of maximal tissue response
RT-PCR = Real time polymerase chain reaction
RXR = Retinoid X receptor
RyR = Ryanodine receptor
s.e.m = Standard error of the mean
SHR = Spontaneously hypertensive rat
siRNA = Small interfering ribonucleic acid
SK_{ca} = Small conductance calcium sensitive potassium channel
SOD = Superoxide dismutase
TEA = Tetraethylammonium chloride
TMH = Transmembrane helices
TNF = Tumour necrosis factor
TRPA = Transient receptor potential ankyrin channel
TRPC = Transient receptor potential canonical channel
TRPM = Transient receptor potential melastatin channel

TRPML = Transient receptor potential mucolipin channel
TRPP = Transient receptor potential polycystic channel
TRPV = Transient receptor potential vanilloid channel
VOCC = Voltage operated calcium channel
WKY = Wistar Kyoto rat

1. INTRODUCTION

1.1. Cannabinoid Ligands and Receptors

The therapeutic history of *Cannabis sativa* is well reviewed by Zuardi (2006). In brief, since the BC era, cannabis has been used as a medicinal plant treating pain, constipation, malaria, convulsions and inflammation (Zuardi 2006). For several years, it was thought that the chemical constituents of *Cannabis sativa* (cannabinoids) caused their effects through non-specific actions on the body (Lawrence and Gill 1975). However, breakthroughs in cannabinoid pharmacology started to occur as early as 1930 (see Pertwee 2006 for review). The most notable discoveries being the isolation and stereochemistry of the plant-derived cannabinoid Δ^9-THC (Gaoni and Mechoulam 1964) and the isolation of the internally synthesised cannabinoid (endocannabinoid), *N*-arachidonoylethanolamine (anandamide, AEA) (Devane *et al.*, 1992). Alongside this, two seven transmembrane G protein-coupled receptors (GPCRs) with cannabinoid specificity have been discovered and fully characterised. The cannabinoid receptor one (CB_1) was first cloned in 1990 and the cannabinoid receptor two (CB_2) was cloned in 1993 (see Pertwee 2006). CB_1 receptors are present in the central nervous system and a range of peripheral tissues, while CB_2 expression is seen in a variety of immune cells and under some conditions in neurons (reviewed in Mackie 2008). To date, over 60 cannabinoid ligands have been isolated from the cannabis plant and there have been many endogenous cannabinoids or cannabinoid-like mediators discovered. There is also speculation of the existence of as yet uncharacterised receptors or subtypes of existing cannabinoid receptors.

1.1.1. Cannabinoid Ligands

A summary of cannabinoid ligands in order of their affinity for CB_1 and CB_2 receptors and cannabinoid chemical structures can be seen in table 1.1 and figure 1.1 respectively.

Phytocannabinoids and Synthetic Cannabinoids

Plant-derived cannabinoids (phytocannabinoids) and synthetic cannabinoids are divided into several groups based on their chemical structures, these include; classical, non-classical, aminoalkylindoles, diarylpyrazoles (for review see Howlett *et al.*, 2002; Pertwee 2005) and

bicyclic sesquiterpene compounds that are part of the essential oil groups found in *cannabis sativa* (Gertsch et al., 2008). Classical phytocannabinoids such as Δ^9-THC and cannabidiol (CBD) are ABC-terpenoid compounds with a functional benzopyran group. Non-classical cannabinoids such as CP55,940 and HU-308 (both synthetic ligands) are either AD-bicyclic or ACD-tricyclic compounds that lack the pyran B ring. Aminoalkylindole cannabinoids are structurally very different to all other groups of cannabinoids and include the synthetic compounds WIN55,212-2 and AM630 (CB_2 antagonists/inverse agonist). AM251, rimonabant and SR144528 are part of the diarylpyrazole family of cannabinoids and are classified as cannabinoid antagonists/inverse agonists due to their reported ability to inhibit endocannabinoid tone or produce effects inverse to cannabinoid receptor agonists (Howlett *et al.*, 2002; Pertwee 2005). The bicyclic sesquiterpene group of cannabinoids include α-humulene and (E)-β-caryophyllene (β-CP) or its isomers (Gertsch *et al.*, 2008). α-humulene has been shown to be void of CB_1 and CB_2 activity, whilst β-CP is considered a natural agonist at the CB_2 receptor (Gertsch *et al.*, 2008).

Several pharmacophores have been identified in determining cannabinoid ligand affinity for cannabinoid receptors. For example, alterations in the C-3 lipophilic alkyl side chain (Huffman *et al.*, 2003), the northern aliphatic hydroxyl group (Mechoulam *et al.*, 1988) and the phenolic hydroxyl group (Huffman *et al.*, 2002) are all capable of altering classical and non-classical cannabinoid receptor affinity. Similarly, alterations in the indole ring of the aminoalkylindole group have been shown to affect cannabinoid receptor specificity of AM630 (Pertwee *et al.*, 1995), and the N-1 and C-5 substituent of the diarylpyrazoles, rimonabant and AM251 (both CB1 receptor antagonists/inverse agonists), are integral to their ability to bind to cannabinoid receptors (Lan *et al.*, 1999).

Cannabinoid ligand affinity and efficacy at cannabinoid receptors has been reviewed in depth elsewhere (Pertwee 2005; Razdan 2009; Vemuri and Makriyannis 2009). However in brief, CP55,940 and WIN55,212-2 are considered full agonists at both CB_1 and CB_2 receptors. HU-308 is a full agonist at CB_2 receptors, whilst THC is a partial agonist at both CB_1 and CB_2 receptors and cannabidiol displays low affinity for both CB_1 and CB_2 receptors. However, these findings

often depend on the assay or preparation used, for example in some preparations, Δ^9-THC, CBD and Δ^9-tetrahydrocannabivarin have been shown to have altered activity at CB_1 receptors depending on ligand concentration (reviewed by Pertwee 2008). The cannabinoid receptor antagonists rimonabant, SR144528, AM251 and AM630 bind to and antagonise cannabinoid receptors at nanomolar concentrations, whilst at higher concentrations they have been shown to have off target effects (discussed section 2.7)

Endocannabinoids

Since the isolation of AEA, several other endocannabinoid ligands have been discovered. Shortly after the isolation of AEA it was discovered that a known endogenous metabolite, 2-arachidonoylglycerol (2-AG), displayed affinity for cannabinoid receptors (Mechoulam *et al.,* 1995; Sugiura *et al.,* 1995). AEA and 2-AG are considered to be the main endocannabinoids, however a range of endocannabinoids or endocannabinoid-like ligands have been discovered, including noladin ether, virodhamine, *N*-arachidonoyl-dopamine (NADA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) (see Bradshaw and Walker 2005; Di Marzo 2009 for review). AEA and 2-AG are thought to be biosynthesised on demand from a series of phospholipid metabolic reactions (for review see Matias and Di Marzo 2007; Muccioli 2010).

The synthesis of AEA requires N-arachidonoyl phosphatidylethanolamine (NAPE), a lipid membrane derivative, formed through Ca^{2+}-dependent and Ca^{2+}-independent *N*-acyltransferase (NAT/iNAT) (Astarita *et al.,* 2008; Jin *et al.,* 2009). NAPE is then hydrolysed to *N*-acylethanolamine through either NAPE-PLD (Wang *et al.,* 2008), GP-NAE (Simon and Cravatt 2006) or a phospholipase C-dependent pAEA pathway (Liu *et al.,* 2006). The major synthetic pathway of 2-AG is through phospholipase C (PLC) hydrolysing arachidonic acid to form diacylglycerol (DAG). DAG is then hydrolysed via DAG lipase alpha and beta (DAGLα/β) forming 2-AG (Stella *et al.,* 1997; Bisogno *et al.,* 2003). Other pathways have been shown to exist in the formation of 2-AG including G_q-receptor-dependent activation of PLCβ1 (Hashimotodani *et al.,* 2005) and phospholipase A_1 leading to lysophosphatidylinositol (LysoPI) being hydrolysed by a LysoPI-specific PLC (Sugiura *et al.,* 1995) (Figure 2). The endocannabinoids OEA and

PEA are also *N*-acylethanolamine derivatives and therefore follow similar synthetic pathways as AEA (Hansen and Diep 2009). However, the endogenous synthesis of the cannabinoid and vanilloid receptor agonist NADA is speculated to involve condensation of dopamine with arachidonic acid (AA) (Huang *et al.,* 2002).

Once formed, endocannabinoids are chemically similar to eicosanoids based on their four non-conjugated *cis* double bonds (Khanolkar and Makriyannis 1999). Endocannabinoids have two pharmacophores; a polar head region and a hydrophobic arachidonoyl side chain (Vemuri and Makriyannis 2009) (Figure 1). AEA and 2-AG levels are reported at low nM concentrations in human plasma (Quercioli *et al.,* 2011), and have been suggested to modulate receptor and target site activity in an autocrine or paracrine manner (Howlett *et al.,* 2011).

Table 2.1. Cannabinoid ligands in order of CB_1/CB_2 affinity

Cannabinoid ligands in order of affinity for CB_1 receptor	CP55,940>AM251>Rimonabant>WIN55212-2>THC >AEA>2-AG>CBD>AM630>HU-308≥β-CP
Cannabinoid ligands in order of affinity for CB_2 receptor	CP55,940>WIN55212-2>HU-308>AM630> THC>AEA>β-CP>Rimonabant>2-AG>AM251>CBD

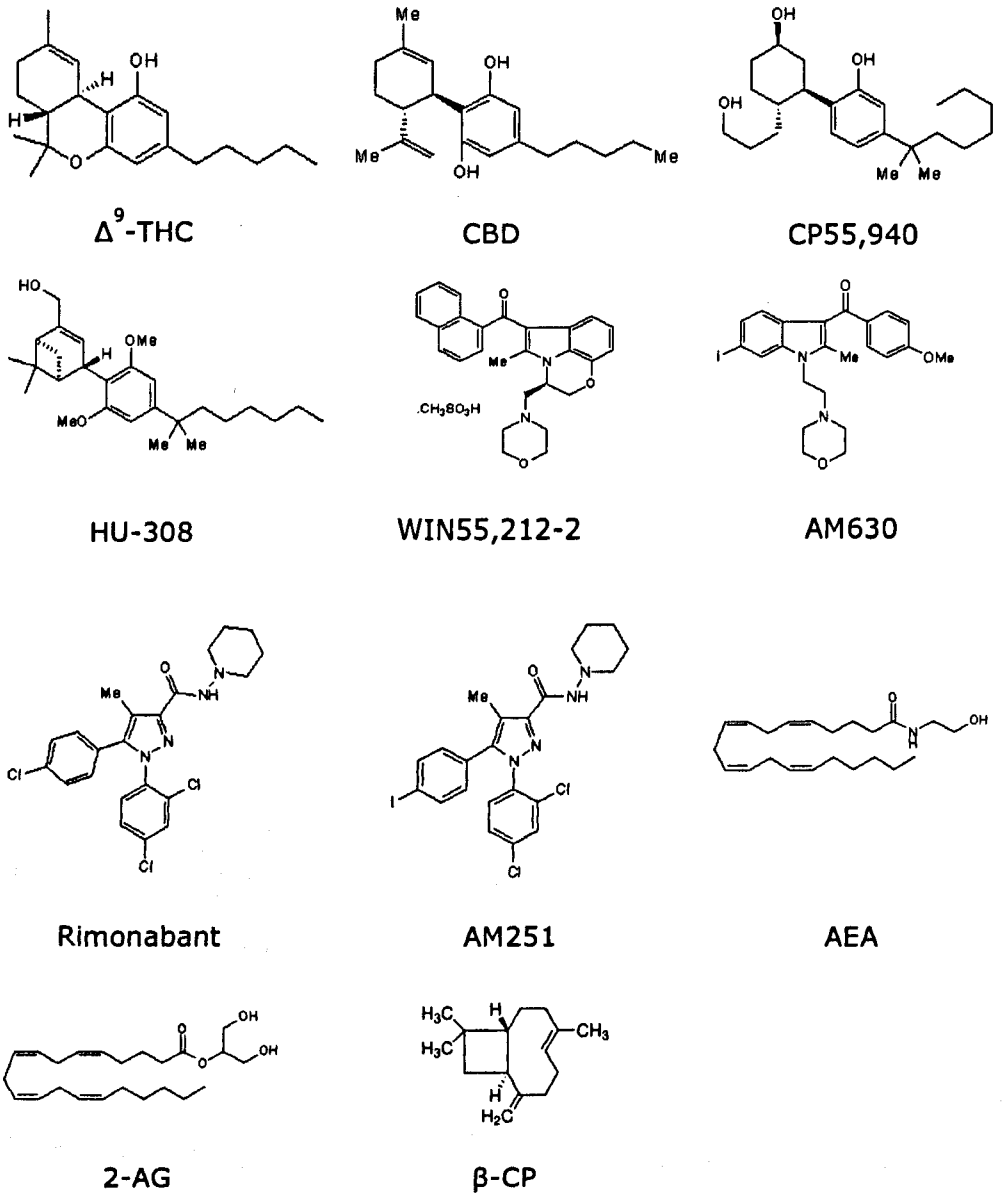

Figure 2.1. Cannabinoid chemical structures
Chemical structures of classical cannabinoids (THC and CBD), non-classical cannabinoids (CP55,940 and HU-308), aminoalkylindoles (WIN55,212-2 and AM630), diarylpyrazoles (Rimonabant and AM251), two endogenous cannabinoids (AEA and 2-AG) and a bicyclic sesquiterpene (β-CP). Chemical structure taken from www.Tocris.com and www.sigmaaldrich.com.

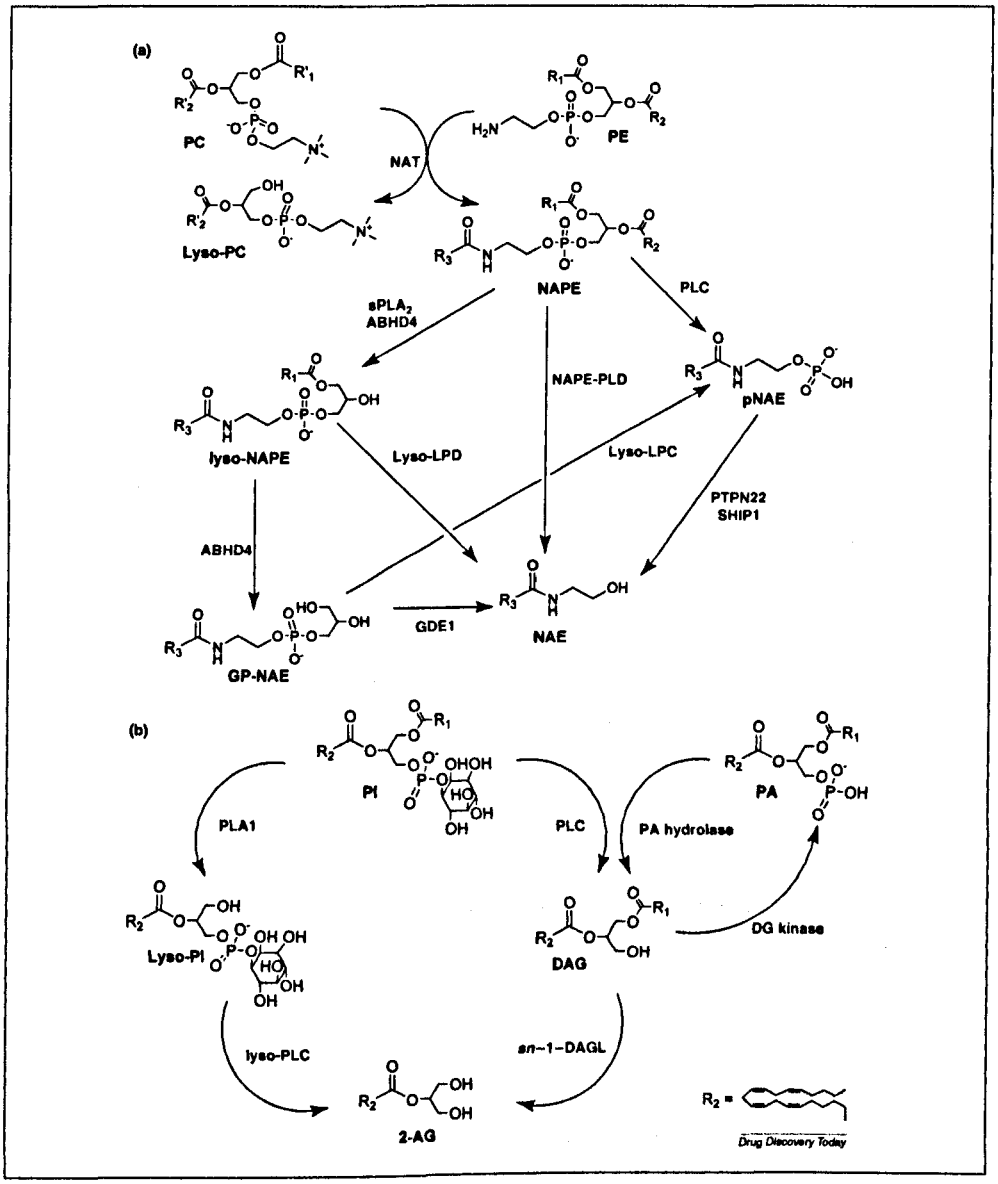

Figure 2.2. Endocannabinoid synthesis.
AEA (a) and 2-AG (b) synthetic pathways adapted from Muccioli (2010). PC, phosphatidylcholine; lyso-PLC, lysophosphatidylinositol-selective phospholipase C; PE, phosphatidylethanolamine; NAT, *N*-acyltransferase; NAPE, N-arachidonoyl phosphatidylethanolamine; sPLA2, secreted phospholipase A2; PLC, phospholipase C; NAPE-PLD, N-acylphosphatidylethanolamine-selective phospholipase D; lyso-NAPE, lysophosphatidyl-NAPE; pNAE, phospho-NAE; NAE, N- acylethanolamine; DAG, diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PLA1, phospholipase A1; PTPN22, phosphatase protein tyrosine phosphatase 22.

1.1.2. Cannabinoid Metabolism

Metabolism of phytocannabinoids has primarily focused on the metabolism of the three main constituents of cannabis, THC,

cannabinol (CBN) and CBD. These compounds are primarily metabolised in the liver via the cytochrome P450 family of enzymes (Yamamoto *et al.,* 1995; Watanabe *et al.,* 2007). Other sites of metabolism have also been reported including the intestine, brain and lung (for review see Pertwee and Huestis 2005). The effect of phase one metabolism on THC, but not other phytocannabinoids, has been well studied. THC undergoes phase one metabolism to produce several metabolites, the main being an 11-hydroxy derivative of THC (11-OH-THC) (Glaz-Sandberg *et al.,* 2007; Ferreiros *et al.,* 2013). 11-OH-THC retains pyschoactivity but is oxidised to 11-nor-Δ^9-carboxy-tetrahydrocannabinol, a non-psychoactive cannabinoid metabolite, and then undergoes phase two metabolism (Glaz-Sandberg *et al.,* 2007). However, the systemic effect of phytocannabinoid metabolites remains to be studied.

Intracellularly, AEA is rapidly degraded by the enzyme fatty acid amide hydrolase (FAAH) (Deutsch and Chin 1993; Cravatt *et al.,* 1996). AEA (and other NEA-derived endocannabinoids) are raised 15- fold in FAAH knockouts compared to wild type controls (Cravatt *et al.,* 2001), suggesting FAAH as a primary enzyme involved in AEA (and potentially other NEA-derived endocannabinoids) metabolism. A second FAAH, FAAH-2 has also been identified in cystolic lipid droplets with a similar catalytic ability to that of FAAH (Wei *et al.,* 2006; Kaczocha *et al.,* 2010). Other pathways for the degradation of NEAs have also been shown through N-acylethanolamine-hydrolysing acid amidase (NAAA) (Tsuboi *et al.,* 2005), however this pathway is pH-dependent and may be more important in the metabolism of other endocannabinoids (Ueda *et al.,* 2010). In all the aforementioned pathways, metabolism of AEA ultimately leads to the production of arachidonic acid and subsequent metabolism through cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP450). However, direct metabolism of AEA through these pathways has also been reported to form prostamides, hydroxyeicosatetraenoic (HETE-ethanolamides) and epoxyeicosatrienoic (EET-ethanolamides) acid-ethanolamides (Bornheim *et al.,* 1995; Ueda *et al.,* 1995; Yu *et al.,* 1997; Snider *et al.,* 2007).

Monoacylglycerol lipase (MAGL) was first suggested to be involved in 2-AG metabolism by Goparaju *et al.,* (1998). This was later confirmed when increases in 2-AG were seen when the genes that code

for MAGL were silenced, confirming MAGL as the primary pathway for degradation of 2-AG (Dinh *et al.,* 2004). However, there is also a role for FAAH metabolism of 2-AG. In cells, Di Marzo *et al.* (1998) have shown, that 2-AG metabolism can be inhibited by methylarachidonylfluorophosphonate (MAFP) and AEA suggesting competition for the same enzyme. In the rodent brain, it has also been shown that α-β-hydrolase domain 6 (ABHD6) and α-β-hydrolase domain 12 (ABHD12) are responsible for a small proportion of 2-AG degradation (Blankman *et al.,* 2007). There is potential for ABHD6 and ABHD12 enzymes to play a role in 2-AG degradation in the periphery, however as yet the research focus is centred upon their role in brain tissue (Savinainen *et al.,* 2012). Metabolism of 2-AG through the aforementioned pathways liberates arachidonic acid and glycerol, of which the arachidonic acid is subsequently metabolised by COX, LOX or CYP450. However, as with AEA, 2-AG can also be directly metabolised through these pathways to produce prostanoids glycerol esters, HETE-glycerol esters and EET-glycerol esters (Kozak *et al.,* 2002; Awumey *et al.,* 2008).

1.1.3. Cannabinoid Target Sites

Cannabinoid Receptors (CB_1)

CB_1 receptors are GPCRs that predominantly signal through $G_{i/o}$ protein coupling and subsequent inhibition of adenylate cyclase (Howlett and Fleming 1984; Howlett *et al.,* 1986), cyclic adenosine monophosphate (cAMP) (Felder *et al.,* 1995), protein kinase A (PKA) (Hampson *et al.,* 1995), Ca^{2+} influx through L-, N-, P/Q-, T- and R-type voltage-sensitive channels (Caulfield and Brown 1992; Mackie and Hille 1992; Mackie *et al.,* 1995; Ross *et al.,* 2008) and intracellular calcium release from ryanodine receptors (RyR) (Zhuang *et al.,* 2005). Stimulatory effects through CB_1 $G_{i/o}$ can also be seen to include membrane hyperpolarisations through potassium channel opening (Deadwyler *et al.,* 1995; Hampson *et al.,* 1995; McAllister *et al.,* 1999) and activation of the mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) pathway through a range of intracellular actions (Bouaboula *et al.,* 1995; Galve-Roperh *et al.,* 2002; Davis *et al.,* 2003; Derkinderen *et al.,* 2003). However, under some circumstances, such as in the presence of the dopamine D_2 receptor, the CB_1 receptor can couple to G_s proteins to stimulate cAMP production (Glass and Felder

1997). Also, WIN55212-2 activation of the CB_1 receptor has been shown to increase intracellular calcium through $G_{q/11}$, coupling (Lauckner *et al.*, 2005).

CB_1 receptors are found in high concentrations in various brain tissues including interneurons, pre-terminal axonal segment and the axons themselves (reviewed Mackie 2008). CB_1 receptors have also be shown to be expressed in the periphery including in adipocytes, liver, pancreas, skeletal muscles (reviewed Mackie 2008) and in human smooth muscle and endothelial cells (Sugiura *et al.*, 1998; Liu *et al.*, 2000).

Cannabinoid Receptors (CB_2)

CB_2 receptors are also GPCRs which are negatively coupled to cAMP through $G_{i/o}$ proteins and inhibition of adenylate cyclase (Felder *et al.*, 1995). Also, inhibition of tumour necrosis factor (TNF), Nuclear Factor-κB (NF-κF) and ras homolog family member A (RhoA) pathways have been observed (Rajesh *et al.*, 2007; Rajesh *et al.*, 2008). The CB_2 receptor has stimulatory actions on MAPK, protein kinase B (PKB), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), Raf-1/Erk and Krox 24 (Bouaboula *et al.*, 1996; Sánchez *et al.*, 2003). The CB_2 receptor is not coupled to G_s subunits (Glass and Felder 1997), phospholipase A_2, C or D, calcium (Q-type and I_{Ca}) or G protein-coupled inward rectifying potassium (GIRK) channels (Felder *et al.*, 1995). However, in endothelial cells, CB_2 antagonism attenuates increased PLC-mediated mobilisation of intracellular Ca^{2+} (Zoratti *et al.*, 2003). CB_2 receptors have been shown to be expressed predominantly in immune cells (reviewed Mackie 2008), in some neurons (reviewed Atwood and Mackie 2010) and in vascular smooth muscle and endothelial cells (Rajesh *et al.*, 2007; Rajesh *et al.*, 2008)

Putative Cannabinoid Receptor Located on the Endothelium (CB_e)

There is also evidence implicating a role for a PTX sensitive non-CB_1 or CB_2 receptor in the cardiovascular system (Jarai *et al.*, 1999). Reported agonists include AEA, methanandamide (synthetic stable analogue of AEA) and Abn-CBD. There has been one reported antagonist at this receptor (O-1918). However, little is known about this receptor and the evidence for its existence will be discussed in section 1.3.

G Protein-coupled Receptors Proposed to be Cannabinoid Receptors

GPR55 is an orphan G protein-coupled receptor widely expressed in human tissues including brain, spleen, adipose and several endothelial cell lines (Godlewski *et al.,* 2009). GPR55 was first cloned in 1999 (Sawzdargo *et al.,* 1999), and since then, several studies have debated whether various cannabinoid ligands bind to and activate GPR55. Ryberg *et al.* (2007) showed, in GPR55-transfected human embryonic kidney (HEK) cells, that 2-AG, CP55,940, THC, AM251 and PEA were able to stimulate GTPγS binding. Lauckner *et al.,* (2008), also observed THC, JWH-015, AEA and methanandamide activated GPR55 in GPR55-transfected HEK cells. Finally, in endothelial cell lines transfected with GPR55, AEA and methanandamide increase calcium signalling (Waldeck-Weiermair *et al.,* 2008). However, contention exists as WIN55,212-2, CP55,940, 2-AG, virodhamine and Abn-CBD were not observed to bind to GPR55-transfected HEK cells by Lauckner *et al.* (2008). A further study in GPR55-transfected HEK cells revealed that although AEA and 2-AG were not able to stimulate calcium signalling, AM251 stimulated calcium signalling and CP55,940 competitively antagonised L-α-lyso-phosphatidylinositol (LPI) induced calcium signalling (Henstridge *et al.,* 2009).

GPR55 is not coupled to $G_{i/o}$ proteins, instead strong evidence points towards either $G_{\alpha 13}$ (Ryberg *et al.,* 2007; Henstridge *et al.,* 2009) or G_q (Lauckner *et al.,* 2008; Waldeck-Weiermair *et al.,* 2008) coupling. Activation of this receptor releases intracellular calcium from intracellular stores involving RhoA kinase and PLC, ultimately having nuclear affects through ERK 1/2 and/or nuclear factor of activated T cells (NFAT) (Ryberg *et al.,* 2007; Lauckner *et al.,* 2008; Waldeck-Weiermair *et al.,* 2008; Henstridge *et al.,* 2009). GPR55 has also been reported to inhibit potassium M type current, with this effect speculated to be through activation of G_q receptor coupling (Lauckner *et al.,* 2008).

It is now widely accepted that the main endogenous agonist of GPR55 is LPI and that synthetic cannabinoids cause greater activation of GPR55 than that of endogenous cannabinoids (Henstridge 2012). Some studies also suggest that cannabinoids may act as allosteric modulators of GPR55 (Anavi-Goffer *et al.,* 2012) or that heteromers may form between GPR55 and cannabinoid receptors (Kargl *et al.,* 2012). Interestingly, it has also been reported that GPR55 signalling

may be inhibited by CB_1 signal transduction (Waldeck-Weiermair *et al.*, 2008).

GPR119 was first identified using bioinformatics (Fredriksson *et al.*, 2003). Human expression of GPR119 is predominantly in pancreatic and intestinal tissues (Soga *et al.*, 2005). Yeast cells transfected with human GPR119 have shown that oleoylethanolamide, palmitoylethanolamide, stearoylethanolamide and AEA bind to GPR119 (Overton *et al.*, 2006). However, the synthetic cannabinoids CP55,940, WIN 55,212-2, methanandamide and JWH-133 have shown no affinity to the receptor. Overexpression of the receptor in HEK cells has been associated with increased levels of cAMP through G_s proteins (Chu *et al.*, 2008). There is evidence for modulation of ATP sensitive potassium (K_{ATP}) channels and voltage-dependent Ca^{2+} channels in GPR119-mediated insulin release (Ning *et al.*, 2008).

GPR18 is predominantly expressed in the spleen, thymus and peripheral lymphocytes (Gantz *et al.*, 1997; Kohno *et al.*, 2006). Recent work conducted in the Bradshaw laboratory has shown that *N*-arachidonoyl glycine, AEA, THC and Abn-CBD are able to activate MAPK through this receptor (McHugh *et al.*, 2012). Also, not only are AEA, THC and Abn-CBD agonists at this receptor but their responses can be inhibited using PTX, AM251 and CBD, a profile that would fit that of the putative CB_e receptor (McHugh *et al.*, 2012). However, as yet there are no studies to show the presence of GPR18 in any vascular bed.

Other Receptor Sites (TRP channels)

Transient receptor potential channels are a superfamily of six, six-trans-membrane cation channels; the canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), mucolipin (TRPML) and polycystin (TRPP) families. However, little is known about endogenous activators of these channels (Wu *et al.*, 2010). A range of cannabinoids, including phytocannabinoids and endocannabinoids, have been shown to bind to TRPV1, TRPV2, TRPM8 and TRPA8 (Pertwee *et al.*, 2010).

TRPV1 channels are activated by a range of stimuli including heat (>43°C), low pH and vanilloid compounds such as capsaicin (found in hot chilli peppers) and capsinate (found in bell peppers) (Wu *et al.*, 2010). Activation of TRPV1 by capsaicin involves binding to

TMH 2-3 (Jordt and Julius 2002). AEA has been shown to bind to this receptor in the same manner as capsaicin (Zygmunt *et al.*, 1999; De Petrocellis *et al.*, 2001). However, there is evidence to suggest that AEA binding to the TRPV1 receptor requires facilitated transport across the cell membrane (De Petrocellis *et al.*, 2001). Endocannabinoids, including AEA and NADA, and other endogenous lipids including some AA metabolites have affinity for the TRPV1 receptor (De Petrocellis and Di Marzo 2005). The phytocannabinoids THC, cannabinol (CBN), CBD (Bisogno *et al.*, 2001; Zygmunt *et al.*, 2002), cannabigerol (CBG), cannabigerovarin (CBGV), Δ^9-tetrahydrocannabivarin (THCV) (De Petrocellis *et al.*, 2011) and synthetic AEA analogues (Di Marzo *et al.*, 2001) have also been shown to activate the TRPV1 receptor. However, WIN55,212-2 is reported to have varying effects at TRPV1 (Evans *et al.*, 2007).

Phosphorylation caused by PKC, PKA (Premkumar and Ahern 2000; Bhave *et al.*, 2002; Mohapatra and Nau 2005) and calmodulin-dependent kinase II (Jung *et al.*, 2004) increases the sensitivity of TRPV1. The receptor is desensitised by Ca^{2+}-dependent protein phosphatase 2B (Jung *et al.*, 2004; Mohapatra and Nau 2005) and inhibited by phosphatidyl-inositol-bis phosphate (PIP2) (Chuang *et al.*, 2001). Furthermore, activation of CB_1 receptors and subsequent CB_1 intracellular signalling affects TRPV1 sensitivity (De Petrocellis *et al.*, 2001; Hermann *et al.*, 2003). Activation of TRPV1 channels increases intracellular calcium (Wu *et al.*, 2010) and has been linked to the release of transmitters such as substance P (Vass *et al.*, 2004), acetylcholine (ACh) (Geber *et al.*, 2006) and calcitonin gene-related peptide (CGRP) (Zygmunt *et al.*, 1999).

TRPV2 channels are similar to TRPV1 channels in that they are activated by noxious heat (>53°C) and permeable to calcium (Wu *et al.*, 2010). At present, there is limited research conducted on the ability of cannabinoids to activate TRPV2. However, in TRPV2-transfected HEK293 cells, a range of phytocannabinoids and synthetic cannabinoids has been shown to mobilise calcium currents (Qin *et al.*, 2008). Further experiments revealed that CBD and CBN also caused calcium influx in TRPV2-transfected HEK293 cells using patch clamp techniques and stimulated the release of CGRP release from rat dorsal root ganglia neurons (Qin *et al.*, 2008).

TRPM8 channels are permeable to calcium and function predominantly as cold sensors and in arterial smooth muscles TRPM8 channels regulate vascular tone (Wu *et al.,* 2010). A range of phytocannabinoids, cannabinoid botanical drug substances and endocannabinoids including CBD, CBG, THC, AEA and NADA antagonise TRPM8-mediated calcium influx in TRPM8-overexpressing HEK293 cells (De Petrocellis *et al.,* 2007; De Petrocellis *et al.,* 2008; De Petrocellis *et al.,* 2011). Further research into the effects of cannabinoids at this receptor is limited. However, it has recently been shown that the botanical drug CBD reduces the size of prostate cancer xenograft tumours in mice, and that CBD causes apoptosis of prostate cancer cells lines partly through antagonism of TRPM8 receptors, and subsequent activation of pro-apoptotic pathways (De Petrocellis *et al.,* 2013).

TRPA1 receptors serve primarily as chemosensors and are able to amplify calcium signalling through activation of other channels (Wu *et al.,* 2010). Activators of TRPA1 channels include cinnamaldehyde (found in cinnamon), isothiocyanates (found in mustard oil and wasabi) and acrolein (found in cigarette smoke) (Wu *et al.,* 2010). The phytocannabinoid THC was shown to stimulate calcium currents in TRPA1-overexpressing HEK293 cells (Jordt *et al.,* 2004). Further work showed that WIN55,212-2 was able to stimulate calcium currents and desensitise TRPA1 channels in TRPA1-overexpressing Chinese hamster ovary cells (Akopian *et al.,* 2008). This work was further supported by the finding that WIN55,212-2 was unable to cause desensitisation in TRPA1 neurons that were knocked down by siRNA (Akopian *et al.,* 2008). Recent work has shown that antinociceptive effects of CBD in mouse-tail were inhibited in part by TRPA1 antagonism (Maione *et al.,* 2011). Similarly, intrathecal injection of the phytocannabinoid Δ^9-tetrahydrocannabiorcol causes antinociception in wild type mice that is lost in $TPA1^{-/-}$ mice (Andersson *et al.,* 2011).

PPAR Receptors

Peroxisome proliferator-activated receptors (PPAR) receptors have been well reviewed in Bishop-Bailey (2000). In brief they are nuclear receptors that consist of three main isoforms: α, γ and δ. PPARα is highly expressed in heart, liver, kidney and skeletal muscle. PPARγ has high levels of expression in heart, spleen, kidney, intestine, adrenal

tissue and skeletal muscle. PPARδ is well expressed in the lung, kidney and brain. PPARs form heterodimers with the retinoid X receptor, leading to gene regulation and transcription at PPAR response elements (reviewed in Bishop-Bailey 2000). Cannabinoid binding to PPAR receptors has been associated with conformational changes of nuclear receptors, aiding in transcriptional activation (reviewed by O'Sullivan 2007). A range of cannabinoids have been shown to activate PPAR receptors including AEA, 2-AG, OEA, PEA, THC, CBD, WIN55,212-2 and cannabinoid metabolites (reviewed in O'Sullivan 2007; Pertwee *et al.*, 2010).

OEA mediates activation of gene transcription by PPARα receptors leading to lipolysis through increased expression of fatty acid binding protein and fatty acid translocase (Guzman *et al.*, 2004). Since that study, other cannabinoids have been shown to activate PPARα receptors including THC, OEA, virodhamine, AEA, WIN55,212-2 and noladin ether in HeLa cells transfected with mPPARα–PPAR response element (reviewed in Sun *et al.*, 2006). OEA, through inhibition of NF-kB, reduces infarct size in a mouse cerebral artery occlusion ischaemia reperfusion model in mice expressing PPARα but is ineffective in PPARα$^{-/-}$ mice (Sun *et al.*, 2007). It has also been shown that, partly through PPARα, PEA reduces the inflammatory effects associated with β-amyloid (Aβ) plaque formation on rat astrocytes (Scuderi *et al.*, 2011). However, in a manner unrelated to changes in inflammatory markers PPARα antagonism inhibits the time-dependent vasorelaxant effects of AEA and PEA in bovine ophthalmic arteries (Romano and Lograno 2012). Further experiments revealed that the vasorelaxant effects of AEA and PEA were inhibited by NOS and BK_{ca} channel inhibitors, suggesting that, through PPARα activation, AEA and PEA increased NO and BK_{ca} channel activation (Romano and Lograno 2012).

In the rat aorta, THC causes time-dependent vasorelaxation that is associated with increased superoxide dismutase and inhibited by antagonism of the PPARγ receptor (O'Sullivan *et al.*, 2005). THC was also shown to bind to PPARγ receptors in PPARγ overexpressing HEK293 cells, and that THC stimulated adipocyte differentiation in 3T3L1 fibroblast cells in a manner comparable to that of the PPARγ agonist rosiglitazone (O'Sullivan *et al.*, 2005). Similarly, work by Bouaboula *et al.* (2005) reported that AEA was also able to bind and

activate PPARγ in a range of cells that had been transfected with this receptor. This study went on to show that AEA was also able to stimulate adipocyte differentiation in 3T3L1 fibroblast cells associated with increased expression of aP2, C-EBPa, Acrp30 and lipoprotein lipase (Bouaboula *et al.,* 2005). The endocannabinoids 2-AG and 2-AG ether also activate PPARγ. In human Jurkat T cell lines transfected with PPARγ, 2-AG and 2-AG ether inhibit interleukin-2 secretion (Rockwell *et al.,* 2006). Recently, it has been shown that the endocannabinoid oleamide was able to displace fluorescent ligands from PPARα, PPARβ and PPARγ receptors (Dionisi *et al.,* 2012). In this assay, oleamide was only able to fully displace the fluorescent ligand from PPARγ receptors, however this was at a lower potency than that of the known PPARγ ligand rosiglitazone (Dionisi *et al.,* 2012). In an *in vivo* study, WIN55,212-2 reversed Aβ-induced TNF-α, activated caspase 3 and nuclear NF-kB expression in the rat hippocampus through CB_1, CB_2 and PPARγ receptors (Fakhfouri *et al.,* 2012).

Much less evidence exists concerning the binding of cannabinoids to PPARβ/δ receptors. Fu *et al.* (2003) reported that OEA binds to HeLa cells that are transfected with PPARβ/δ receptors. However, the authors reported that the appetite suppression effects of OEA were unlikely to depend on OEA activation of PPARβ/δ (Fu *et al.,* 2003). Further studies have shown that ajulemic acid, 2-AG metabolites and PEA are all without PPARβ/δ affinity (Kozak *et al.,* 2002; Liu *et al.,* 2003; LoVerme *et al.,* 2006)

1.2. Cannabinoids in the Vasculature

1.2.1. Modulation of Vascular Tone

Arterial tone is modulated through several pathways within smooth muscle and endothelial cells. Sympathetic sensory mechanisms control arterial tone via perivascular nerves. Perivascular nerves extend into arteries forming neuroeffector junctions on vascular smooth muscle cells and are capable of mediating both contraction and dilation (Kreulen 2003). Smooth muscle cells react to neurohormonal stimuli, interact with the endothelium, repair arterial damage and maintain vascular tone (Lacolley *et al.,* 2012). Endothelial cells produce several vasoactive mediators that interact with smooth muscle cells causing vasoconstriction or vasodilatation (Vanhoutte *et al.,* 2009). The main

mediators in vascular tone regulation appear to be NO and potassium channel hyperpolarisation. However, these mediators are often stimulated by physical forces, circulating hormones, platelet-derived products, autacoids and metabolites of arachidonic acid (AA) (Vanhoutte *et al.,* 2009). Cannabinoids have also been shown to mediate vascular tone through a range of mechanisms of action, these mechanisms of action are broadly dependent on several factors including the species, the vascular bed, the differences between the cannabinoid ligands and the method of investigation used. The pathways involved include activation of cannabinoid receptors, activation of TRPV1 receptors, modulation of ion channels, release of endothelium-derived relaxing factors, products of metabolism, activation of novel receptors and activation of PPAR receptors. There are excellent reviews available detailing the effects of cannabinoids in the vasculature (Hillard 2000; Kunos *et al.,* 2000; Randall *et al.,* 2002; Randall *et al.,* 2004). The following sections will take into account studies cited in these reviews plus more recent studies.

1.2.2. *In Vivo* Anesthetised Animal Studies

The first evidence of the vasodilator effects of cannabinoids was shown by measurement of cerebral artery diameter using a cranial window technique in live anesthetised rabbits after topical application of THC and AEA (Ellis *et al.,* 1995). The effects of AEA and THC were insensitive to nitric oxide synthase (NOS) inhibition using N $^{(G)}$-nitro-L-arginine methyl ester (L-NAME) and removal of oxygen free radicals using superoxide dismutase. However, responses to both agonists were inhibited using indomethacin, implicating the role of COX-derived metabolites. In the dural arteries of anaesthetised Sprague-Dawley rats, AEA-induced vasodilation has also been shown to be sensitive to TRPV1 and CRGP receptor antagonism (Akerman *et al.,* 2004). However, it must be noted that a role for COX metabolism was not investigated in this work (Akerman *et al.,* 2004).

Several cannabinoid agonists including THC, HU-210, CP55,940, WIN55,212-2, JWH-015, AEA and methanandamide cause prolonged hypotension in anaesthetised Sprague-Dawley rats, an effect that is sensitive to the CB_1 antagonist rimonabant (Lake *et al.,* 1997; Wagner *et al.,* 2001). However, Lake *et al.,* (1997) reported that the inhibition of AEA by CB_1 antagonism was only at selected time points, unlike the

effects of CB_1 antagonism on the other cannabinoids used in the study. Similarly, in anaesthetised spontaneously hypertensive rats (SHR) and Wistar Kyoto rats, AEA, HU-210 and the FAAH inhibitor URB597 caused reductions in mean arterial pressure, sensitive to rimonabant or AM251 (Batkai *et al.,* 2004). Further support for the role of the CB_1 receptor in the control of systemic blood pressure has been shown in anaesthetised SHR and cirrhotic rats (Batkai *et al.,* 2004; Moezi *et al.,* 2006; Batkai *et al.,* 2007). In these models, it was also shown that a CB_1 antagonist given alone revealed a hypertensive effect (Batkai *et al.,* 2004; Moezi *et al.,* 2006; Batkai *et al.,* 2007). It has also been shown that antagonism of the CB_1 receptor potentiates tachycardia and vasoconstriction associated with myocardial infarction (Rudz *et al.,* 2012). Suggesting that, under the ischaemic conditions associated with myocardial infarction, endocannabinoids through CB_1 activation have both a bradycardic and hypotensive effect.

CB_2 agonists also have hypotensive and vasodilator effects. In anaesthetised Sabra rats, HU-308 caused a decrease in blood pressure that was attenuated using the CB_2 antagonist SR144528 (1 mg/kg^{-1}) and not rimonabant (3 mg/kg^{-1}) (Hanus *et al.,* 1999). JWH-133 and JWH-015 also caused dilation of knee synovial blood vessels measured using laser Doppler imaging in Wistar rats, the effect of JWH-133 was attenuated using AM360 (10 nM) and TRPV1 antagonism (McDougall *et al.,* 2008).

In vivo anaesthetised studies clearly show that cannabinoids are able to cause hypotension in animals. This appears to be primarily through the CB_1 receptor, but also with some involvement from both CB_2 and TRPV1 receptors. Furthermore, the roles of the CB_1 receptor and cannabinoid induced hypotension appear to be more pronounced in disease models.

1.2.3. *In Vivo* Conscious Animal Studies

The *in vivo* effects of cannabinoids in conscious rats differ significantly from their actions in anaesthetised animals. The cannabinoid agonists AEA, WIN55,212-2 and THC have all been shown to cause pressor effects accompanied by renal and mesenteric vasoconstriction and hindquarters vasodilation (Gardiner *et al.,* 2001; Gardiner *et al.,* 2002; Gardiner *et al.,* 2002; O'Sullivan *et al.,* 2007; Wheal *et al.,* 2007; Ho and Gardiner 2009). The responses to WIN55,212 and THC were

antagonised using the CB_1 antagonist AM251 (Gardiner *et al.*, 2002). The pressor response to WIN55,212-2 was also attenuated by antagonising the vasopressin V_1 receptor, angiotensin-II AT_1 receptor and ganglionic blockade (Gardiner *et al.*, 2001). However, the hindquarters vasodilation to WIN55,212-2 was inhibited by β_2 receptor antagonism (Gardiner *et al.*, 2002). In a further study, the hindquarters vasodilation caused by THC was decreased in rats that had been chronically treated by a NOS inhibitor, suggesting that the vasodilation to THC may, in part, be mediated by NO release (O'Sullivan *et al.*, 2007).

AEA (>1.25 mg kg^{-1}) caused a brief systemic pressor effect associated with vasoconstriction of renal and mesenteric vascular beds and vasoconstrictor and then vasodilator effects in the hindquarters (Gardiner *et al.*, 2002). Other works report similarly that AEA (3 mg kg^{-1}) caused a brief constrictor effect in all vascular beds followed by a vasodilation in both the renal and mesenteric vascular beds (Ho and Gardiner 2009). In both studies, the effects of AEA were insensitive to AM251 (3 mg kg^{-1}). Gardiner *et al.* (2002a) report that the hindquarters vasodilation in response to high concentrations of AEA was inhibited using a β_2 antagonist and that the same antagonist prolonged the renal and mesenteric vasoconstriction. Interestingly, FAAH inhibition prolonged the hindquarters vasodilation of high concentrations of AEA, but inhibited hindquarters vasodilation caused by low doses of AEA (Ho and Gardiner 2009).

The effects of cannabinoids in conscious animals differ from those seen in unconscious animals. The above studies have revealed that cannabinoids cause a longer-lasting pressor responses and shorter-lasting decreases in mean arterial pressure than in anaesthetised animals. It has been suggested that in conscious freely-moving animals the cannabinoid response is dependent on the pre-existing level of sympathetic tone, which might be linked to the CB_1 receptors. The above studies reveal methodological differences between assessments of the actions of cannabinoids in the vasculature and could point to a potential role of anaesthesia.

1.2.4. Cannabinoid Receptor Knockout Models

Blood pressure and heart rate were similar in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice (Jarai *et al.*, 1999; Ledent *et al.*, 1999). This might suggest that

these receptors are not involved in blood pressure regulation under normal conditions. In both conscious and anaesthetised $CB_1^{-/-}$ mice, or isolated arteries from $CB_1^{-/-}$ mice, WIN55,212-2, AEA and HU-210 were unable to cause the hypotensive effect that is seen in $CB_1^{+/+}$ mice (Jarai *et al.,* 1999; Ledent *et al.,* 1999; Szekeres *et al.,* 2012). This suggests that the CB_1 receptor is intrinsic to cannabinoid-induced systemic hypotension. However, in an *in vitro* model, topical application of AEA, methanandamide and Abn-CBD caused vasorelaxation in the mesenteric vasculature of $CB_1^{-/-}$ mice (Jarai *et al.,* 1999). This vasorelaxation was sensitive to a relatively high concentration of Rimonabant (1 μM), endothelium denudation, charybdotoxin (ChTX) and apamin. Furthermore, it was insensitive to L-NAME and indomethacin (Jarai *et al.,* 1999). These findings point towards the role of a putative cannabinoid receptor located on the endothelium (CB_e) that is sensitive to rimonabant and potentially capable of ion channel modulation. Later work from the same group has shown that 2-AG-induced hypotension of anaesthetised $CB_1^{+/+}$ and $CB_1^{-/-}$ mice (Jarai *et al.,* 2000). This vasodilation was insensitive to rimonabant (3 mg kg^{-1}) but was inhibited by indomethacin (Jarai *et al.,* 2000), suggesting that the systemic hypotensive effects caused by 2-AG are mediated via COX derived metabolites.

Using CB_2 knockout mice ($CB_2^{-/-}$) there is strong evidence to suggest that the CB_2 receptor is not involved in systemic hypotension (Jarai *et al.,* 1999). Recent reports have also shown $CB_2^{-/-}$ mice share similar cardiovascular and haemodynamic traits as their wild type counterparts (Defer *et al.,* 2009). However, $CB_2^{-/-}$ mice have been associated with decreased protection from cardiovascular and vascular injury (reviewed in Buckley 2008; Pacher and Haskó 2008). Indeed, studies have shown, using $CB_2^{-/-}$ mice, that the effects of atherosclerosis are potentiated in the absence of the CB_2 receptor. Atherosclerotic plaque sizes are reported to remain the same size as wild type, however $CB_2^{-/-}$ mice are associated with increased macrophage and smooth muscle cell infiltration and loss of plaque stability (Netherland *et al.,* 2010) and increased macrophage infiltration with increased aortic superoxide production (Hoyer *et al.,* 2011).

1.2.5. Summary of *In Vivo* Findings

The *in vivo* anaesthetised animal studies have shown cannabinoid-induced hypotension is mediated predominantly via the CB_1 receptor. However, in conscious animals, cannabinoids cause a pressor effect linked to CB_1-mediated sympathoexcitation that may have previously been masked in anaesthetised animals.

1.2.6. Cannabinoid Vascular Actions *In Vitro*

Cannabinoid induced vasorelaxation has been shown in a variety of *in vitro* models via several key mechanisms, which are discussed in the following sections.

CB_1 Receptor

CB_1 receptor mRNA has been detected in rat vasculature endothelial cells (Deutsch *et al.*, 1997). Human vascular expression of CB_1 mRNA has also been shown (Sugiura *et al.*, 1998; Liu *et al.*, 2000). A role for CB_1-mediated vasorelaxation has been implicated in several studies using the CB_1 antagonist rimonabant. However, at high doses rimonabant causes effects that are not selective to the CB_1 receptor (for review see Pertwee 2005). Therefore in the following section, rimonabant and AM251 will only be considered to be antagonising CB_1 receptors at concentrations of ≤ 1 μM.

AEA, methanandamide, noladin ether, HU-210 and CP55,940 all have high affinities at the CB_1 receptor and all cause vasorelaxation that is inhibited by CB_1 antagonism. The vasorelaxant effects of AEA were reduced in rat resistance arteries in the presence of AM251 (O'Sullivan *et al.*, 2004). Similarly, the effects of noladin ether were completely abolished in the presence of AM251 or rimonabant in rabbit pulmonary arteries (Su and Vo 2007). Furthermore, in rat mesenteric resistance arteries, the potency and maximum vasorelaxation to CP55,940 and HU-210 were reduced by rimonabant (White and Hiley 1998).

To further establish the mechanisms behind CB_1-mediated vasorelaxation, White *et al.* (1998) have shown that CP55,940 causes relaxation in rat mesenteric resistance arterial segments contracted using high potassium solution. This effect is sensitive to rimonabant, suggesting that, through a CB_1-mediated pathway, CP55,940 inhibits voltage-operated calcium channels. In vascular smooth muscle, AEA

and WIN 55212-2 are able to cause a decrease in peak Ca^{2+} current in cat cerebral smooth muscle arteries in a rimonabant- and pertussis toxin- (PTX, inhibitor of $G_{i/o}$ proteins) sensitive manner (Gebremedhin *et al.,* 1999). Also, vasorelaxation to AEA in rat coronary arteries was inhibited by rimonabant and calcium dependent potassium channel blockade (Randall and Kendall 1997). In rat aorta, methanandamide-induced vasorelaxation is inhibited by CB_1 and CB_2 antagonism (Lopez-Miranda *et al.,* 2010). This work also shows concurrent roles for cytochrome p450 and guanylate cyclase suggesting roles for metabolism and production of nitric oxide in methanandamide-induced vasorelaxation (Lopez-Miranda *et al.,* 2010).

Su *et al.* (2007) have shown that the vasorelaxation caused by noladin ether in rabbit pulmonary arterial strips is through the CB_1 receptor and associated with MEK/ERK 1/2 activation. This was confirmed using western blotting studies showing that CB_1 antagonism reduced MEK/ERK 1/2 phosphorylation associated with noladin ether.

Interestingly, a recent study has shown that antagonism of the CB_1 using O2050, a neutral CB_1 antagonist as well as AM251 and rimonabant, augments the vasoconstrictor effects of angiotensin II (Szekeres *et al.,* 2012). In the same work it is also reported that inhibition of 2-AG synthesis also augments angiotensin II responses, suggesting that 2-AG through CB_1 activation may counteract vasoconstriction (Szekeres *et al.,* 2012).

CB_2 Receptor

CB_2 receptor mRNA has been shown in the superior mesenteric arteries of rats (Moezi *et al.,* 2006). Immunohistochemical analysis of human cerebral endothelial cells has confirmed that 45% cells were positive for the CB_2 receptor (Schley *et al.,* 2009). In rat mesenteric resistance arteries, the CB_2 receptor agonist JWH-015 causes vasorelaxation (Ho and Hiley 2003). However, the effects of JWH-015 were not inhibited by CB_2 antagonism using SR144528, shedding doubt on the role for CB_2. In the rat aorta, the vasorelaxant effect of the potent CB_1 and CB_2 agonists CP55,940 and WIN55,212-2 were partially inhibited by CB_2 antagonism using SR144528 (O'Sullivan *et al.,* 2005; Dannert *et al.,* 2007). In rat coronary arteries, it has been shown that AEA and HU-210 induced vasorelaxation that was antagonised using AM630 (Mair *et al.,* 2010). This work also showed that the effects of AEA were

not inhibited by removal of the endothelium, but were inhibited by the non-selective COX inhibitor indomethacin and the sphingosine kinase inhibitor SKi (Mair *et al.,* 2010). The intracellular signalling coupled to the CB_2 receptor regarding vasorelaxation has not been fully explored however the previous study suggests that CB_2 stimulation may lead to ERK1/2 phosphorylation, production of arachidonic acid (leading to the production of vasorelaxant prostaglandins) and production of sphingosine-1-phosphate (leading to activation of endothelial $S1P_3$ receptors).

TRPV1 Receptor

Zygmunt *et al.* (1999) first showed that the vasorelaxant effects of AEA, but not 2-AG, PEA, HU-210, WIN55,212-2 and CP55,940, could be antagonised by the TRPV1 antagonist capsazepine in rat mesenteric arteries. AEA caused vasorelaxation through TRPV1 stimulation of sensory nerves to release CRGP, which was further confirmed by inhibition of vasorelaxation through CRGP antagonism using $CGRP_{8-37}$ (Zygmunt *et al.,* 1999). However, AEA-induced vasorelaxation though the TRPV1 receptor has also been reported to be linked to stimulation of nitric oxide production in the rat mesenteric vascular bed (Poblete *et al.,* 2005). Similarly, vasorelaxation to methanandamide in rabbit aortic endothelium denuded rings was also completely inhibited by capsazepine and $CGRP_{8-37}$ (Mukhopadhyay *et al.,* 2002). Vasorelaxation caused by the endocannabinoid NADA in the rat superior mesenteric artery is inhibited by desensitisation of the TRPV1 receptor using capsaicin and antagonism using capsazepine (O'Sullivan *et al.,* 2004). However, in rat coronary arteries and rat pulmonary arteries AEA-induced vasorelaxation was not affected by incubation with capsaicin and capsazepine (White *et al.,* 2001; Baranowska-Kuczko *et al.,* 2012). Phytocannabinoids or their analogues have also been shown to cause TRPV1-mediated vasorelaxation. In rat hepatic arteries, THC and cannabinol-induced vasorelaxation are inhibited by TRPV1 receptor desensitisation using capsaicin and antagonism of $CGRP_1$ receptors using $CGRP_{8-37}$ (Zygmunt *et al.,* 2002). Interestingly, in mesenteric arteries from TRPV1 knockout mice, the vasorelaxation to AEA is almost completely abolished, however the vasorelaxation to THC is only slightly reduced (Zygmunt *et al.,* 2002). This suggests that the TRPV1 receptor is the main mechanism involved in AEA-induced

vasorelaxation, whereas TRPV1 only partially mediated the effects of THC. However, in rat mesenteric resistance arteries, the effects of THC are unaffected by capsaicin pre-treatment, capsazepine and ruthenium red (O'Sullivan *et al.,* 2005). In the rat aorta, vasorelaxation to WIN55,212-2 was partially inhibited via TRPV1 desensitisation or antagonism (Dannert *et al.,* 2007).

Ion Channels

In rat mesenteric arteries and rat coronary arteries, the vasorelaxant effects of AEA were inhibited using non-specific potassium channel inhibitors and BK_{ca} channel inhibitors (Plane *et al.,* 1997; White *et al.,* 2001). A novel endogenous ligand chemically similar to AEA, *N*-arachidonoyl $_L$-serine (ARA-S) has also been shown to directly activate BK_{Ca} channels in rat mesenteric arteries which was confirmed by patch clamping in HEK cells (Godlewski *et al.,* 2009). In rat mesenteric resistance arteries, JWH-015, WIN55,212-2 and WIN55,212-3 produced vasorelaxation of methoxamine-constricted arterial segments that was insensitive to CB_1 and CB_2 antagonism (Ho and Hiley 2003). However, when calcium chloride was used as the contractile agent vasorelaxation to JWH-015, WIN55,212-2 and WIN55,212-3 was reduced, as was vasorelaxation to verapamil (L-type calcium channel blocker) (Ho and Hiley 2003), suggesting a direct inhibition of calcium influx. OEA inhibits increased perfusion pressure caused by caffeine-induced intracellular calcium release in the rat perfused mesenteric arterial bed *in situ* (Wheal *et al.,* 2010). This inhibition was not sensitive to CB_1 antagonism, suggesting the direct inhibition of release of intracellular calcium.

The Endothelium, Nitric Oxide and EDHF

The endothelium has a substantial role in the regulation of vascular tone (reviewed in Feletou and Vanhoutte 2009). Vasorelaxation caused by AEA is inhibited by endothelial removal in bovine ophthalmic arteries (Pratt *et al.,* 1998), rat first and third order mesenteric arteries (O'Sullivan *et al.,* 2004; Ho and Randall 2007; Wheal *et al.,* 2012), rabbit aortic rings (Mukhopadhyay *et al.,* 2002) and rabbit superior mesenteric arteries (Chaytor *et al.,* 1999). 2-AG-, oleamide-, OEA- and NADA-induced vasorelaxation in the rat mesenteric resistance artery is also inhibited by removal of the endothelium (O'Sullivan *et al.,* 2004;

Ho and Randall 2007; Sudhahar *et al.*, 2009; Wheal *et al.*, 2012). Methanandamide-induced vasorelaxation is also inhibited in rabbit aortic ring endothelium-denuded arterial segments (Mukhopadhyay *et al.*, 2002).

NO is an endothelium-derived factor capable of causing vascular hyperpolarisation (reviewed in Feletou and Vanhoutte 2009). Nitric oxide inhibition inhibits vasorelaxation to AEA in the rat renal vasculature (Deutsch *et al.*, 1997), AEA and WIN55,212-2 in the bovine ophthalmic arteries (Romano and Lograno 2006), OEA in the rat isolated mesenteric bed (Wheal *et al.*, 2010), AEA (first order) and OEA (third order) in isolated mesenteric arteries (Wheal *et al.*, 2012) and methanandamide in rabbit aortic rings (Mukhopadhyay *et al.*, 2002). However, in the rat and rabbit mesenteric resistance and rat conduit arteries, nitric oxide inhibition does not inhibit AEA-, NADA-, THC-, cannabinol- and Abn-CBD-induced vasorelaxation (Chaytor *et al.*, 1999; Zygmunt *et al.*, 2002; Offertaler *et al.*, 2003; O'Sullivan *et al.*, 2004; O'Sullivan *et al.*, 2004).

Endothelium-derived hyperpolarising factor (EDHF), involves activation of small- (SK_{Ca}) and intermediate- (IK_{Ca}) conductance calcium activated potassium channels (Xia et al 1998; Khanna *et al* 1999). This activation leads to increases in intracellular Ca^{2+} in the endothelium and K^+ efflux. The efflux of K^+ leads to myocyte hyperpolarisations through inwardly rectifying potassium channels and sodium potassium ATP-driven exchangers (reviewed in Edwards *et al.*, 2010). A role for EDHF vasorelaxation has been implicated in cannabinoid-induced vasorelaxation. EDHF-mediated relaxation has been implicated in AEA-induced vasorelaxation of rat arteries (Randall *et al.*, 1996; Randall *et al.*, 1997; O'Sullivan *et al.*, 2004). Recent work has also shown that EDHF-mediated vasorelaxation of AEA and OEA in rat mesenteric arteries may involve liberation of H_2O_2 as both AEA and OEA relaxations are inhibited by catalase (Wheal *et al.*, 2012).

Metabolism

Vasoactive metabolites of AEA and THC have been shown to cause vasodilation in cerebral arterioles sensitive to the COX inhibitor indomethacin (Ellis *et al.*, 1995). In rat skeletal arteries, AEA-induced vasorelaxation is inhibited by abolishing AEA breakdown through FAAH, COX and cytochrome P450 (Czikora *et al.*, 2012). However, in rat

renal, mesenteric resistance and conduit arterial segments, the effects of AEA are not inhibited by indomethacin (Deutsch *et al.*, 1997; O'Sullivan *et al.*, 2004). The role for metabolism may be dependent on the cannabinoid in question. 2-AG is reported to be an unstable endocannabinoid readily susceptible to metabolism (Rouzer *et al.*, 2002). Endothelium-dependent metabolism of 2-AG by FAAH, MAGL and COX are responsible for 2-AG-induced vasorelaxation of bovine coronary arteries (Gauthier *et al.*, 2005) or metabolism through CYP450 in rat mesenteric arteries (Awumey *et al.*, 2008). However, metabolism of 2-AG is not implicated in causing 2-AG-induced vasorelaxation of the rabbit mesenteric artery (Kagota *et al.*, 2001) or rat mesenteric arteries (Ho and Randall 2007). Interestingly however, it has been shown that COX-1 inhibition potentiates the vasorelaxation seen to OEA and 2-AG, whereas inhibition of COX-2 potentiates vasorelaxation seen to AEA in perfused *in situ* rat mesenteric arteries and isolated mesenteric arteries (Ho and Randall 2007; Wheal *et al.*, 2010).

Non-CB_1, Non-CB_2 Vascular Receptors

Early indications of a novel vascular cannabinoid receptor came from the works of Jarai *et al.* (1999), which showed that in $CB_1^{-/-}$, $CB_2^{-/-}$ and wild type mice, AEA, methanandamide and Abn-CBD were able to cause vasodilation of the mesenteric vasculature (Jarai *et al.*, 1999). The vasodilation was inhibited by removal of the endothelium and the presence of rimonabant suggesting that rimonabant has inhibitory affects at the CB_e receptor. Further work has shown Abn-CBD caused vasorelaxation of the rat mesenteric artery, this relaxation is sensitive to endothelium denudation, O-1918 (a proposed antagonist at the CB_e receptor that has no affinity at CB_1 or CB_2 receptors) and PTX (Offertaler *et al.*, 2003). This finding, as with that of Jarai *et al.* (1999b), suggests that the relaxation seen to Abn-CBD is mediated by an endothelial $G_{i/o}$ protein-coupled receptor that is neither the CB_1 nor CB_2 receptor. Experiments in the rabbit pulmonary artery have shown also that Abn-CBD causes relaxation that is only partially inhibited by CB_1 antagonism, yet fully inhibited by O-1918 (Su and Vo 2007). In rabbit aortic rings, AEA and methanandamide also cause vasorelaxation through the novel PTX-sensitive endothelial receptor (Mukhopadhyay *et al.*, 2002). Studies in rat aorta have shown that AEA causes

endothelium-dependent vasorelaxation that is sensitive to PTX but insensitive to rimonabant (1 μM) (Herradón *et al.*, 2007). Oleamide is also sensitive to PTX, O-1918 and antagonism of calcium-activated potassium channels but not 1 μM AM251 in rat mesenteric resistance arteries (Hoi and Hiley 2006). Abn-CBD modulates BK_{Ca} channel currents in human umbilical endothelial cells in patch clamp studies. This modulation was insensitive to CB_1/CB_2 receptor antagonism, but was inhibited using PTX or O-1918 (Begg *et al.*, 2003). These findings offer further support for the presence and vasorelaxant role of the CB_e receptor, and suggest it is $G_{i/o}$ protein coupled, capable of activating calcium-operated potassium channels and antagonised by both O-1918 and rimonabant.

The vasorelaxant effects of NADA and AEA in the rat aorta have been shown to be unaffected by AM251 (1 μM) but are partly sensitive to PTX (O'Sullivan *et al.*, 2005). However, removal of the endothelium did not affect the response to AEA or the maximum response to NADA (O'Sullivan *et al.*, 2005). In rat mesenteric arteries, the endothelium-independent vasorelaxant effects of THC are mediated through a $G_{i/o}$ protein, potentially coupled to K^+ and Ca^{2+} channels (O'Sullivan *et al.*, 2005). These findings potentially suggest a further $G_{i/o}$ protein-coupled receptor located on the smooth muscle that also has the ability to modulate ion channels. This receptor differs from CB_e receptor, as the CB_e receptor is antagonised by rimonabant in mesenteric resistance arteries (Jarai *et al.*, 1999).

Evidence suggests GPR55 is not the endothelial cannabinoid receptor, as mesenteric arteries of wild type and GPR55 knockout mice show vasorelaxation in the presence of Abn-CBD and O-1602 (an analogue of Abn-CBD), which is sensitive to O-1918 (Johns *et al.*, 2007). Furthermore, the pharmacological profile of the proposed endothelial cannabinoid receptor differs from the pharmacological profile of GPR55, as GPR55 is either $G_{\alpha 13}$ or G_q coupled and the CB_e receptor is $G_{i/o}$ coupled (Offertaler *et al.*, 2003; Waldeck-Weiermair *et al.*, 2008; Henstridge *et al.*, 2009).

PPAR Receptors

The first report to show that cannabinoids modulate vascular tone through interactions with the PPAR receptors came from the work of O'Sullivan *et al.* (2005). This study showed that THC (10 μM) caused a

time-dependent (over a period of 120 minutes) vasorelaxation in pre-constricted rat aorta. The maximum vasorelaxant effect of THC was similar to that of the PPARγ agonist rosiglitazone, with both effects being inhibited by the PPARγ selective antagonist GW9662 (O'Sullivan *et al.,* 2005). The effects of THC were also inhibited by the addition of cycloheximide, endothelium denudation, L-NAME, catalase and SOD inhibition (O'Sullivan *et al.,* 2005). These findings suggested that THC induced vasorelaxation through activation of PPARγ receptors and subsequent increases in NO bioavailability (O'Sullivan *et al.,* 2005). Furthermore, after two hours incubation with THC, the superior mesenteric artery shows enhanced ACh responses that was blocked by PPARγ antagonism (O'Sullivan *et al.,* 2006). PPARγ-mediated vasorelaxation in rats seems to be exclusive to the conduit arteries as PPARγ-mediated vasorelaxation in the mesenteric resistance arteries of rats was not observed (O'Sullivan *et al.,* 2005; O'Sullivan *et al.,* 2006).

CBD, NADA and AEA have also been shown to cause time-dependent relaxation that was inhibited by antagonism/inhibition of PPARγ, SOD, NO and protein synthesis (O'Sullivan *et al.,* 2009; O'Sullivan *et al.,* 2009). However, the effects of both NADA and AEA were partially dependent on the endothelium (O'Sullivan *et al.,* 2009). This could suggest that NADA and AEA could have activated the smooth muscle PPARγ receptor. NADA was also found to be dependent on the CB_1 receptor. The authors concluded that NADA may activate the PPARγ receptor via a CB_1-dependent mechanism, potentially through the ERK1/2 and MAPK (O'Sullivan *et al.,* 2009)

Recent research has shown in the bovine ophthalmic artery that AEA and PEA cause vasorelaxation through a PPARα-mediated pathway (Romano and Lograno 2012). The vasorelaxation observed was not dependent on the presence of the endothelium, PPARγ antagonism or TRPV1 desensitisation (Romano and Lograno 2012). However, vasorelaxation was inhibited by PPARα antagonism, nitric oxide synthase inhibition and blocking of BK_{Ca} channels (Romano and Lograno 2012).

1.2.7. Summary of *In Vitro* Findings

In vitro studies have shown that cannabinoids produce vasorelaxation through a range of mechanisms including activation of CB_1 receptors, CB_2 receptors, TRPV1 receptors, sensory nerves, novel cannabinoid

receptors, endothelium-derived relaxant products including EDHF, metabolism, modulation of ion channels and interaction with PPARα and PPARγ nuclear receptors. These mechanisms of action are dependent on the cannabinoid ligand used and the vascular bed studied.

1.2.8. Cannabinoids in Cardiovascular Disease

Heart Disease

There is a range of evidence to support a role for the CB_2 receptor in cardio-protection. Early work showed that LPS pre-treatment reduced infarct size caused by ischaemia/reperfusion injury in isolated rat hearts (Lagneux and Lamontagne 2001). However, the protective effects of LPS were inhibited when hearts were perfused with a CB_2 antagonist or a NOS inhibitor (Lagneux and Lamontagne 2001). Similarly, in isolated rat hearts, heat stress prior to ischaemia/reperfusion injury caused a reduction in infarct size, in a manner that was dependent on the CB_2 receptor and NO (Joyeux *et al.*, 2002). Further studies then explored potential roles for endocannabinoid signalling in cardio-protection by pre-treating hearts with endocannabinoids prior to ischaemia/reperfusion. In hearts that were infused with 2-AG and PEA for 15 minutes prior to ischemia/reperfusion a reduced infarct size was observed (Lepicier *et al.*, 2003). The cardio-protective effects of 2-AG and PEA were inhibited by CB_2 antagonism or PKC, MAP kinase or ERK1/2 inhibition (Lepicier *et al.*, 2003). The synthetic CB_2 agonist JWH-133 when perfused 5 minutes prior to reperfusion was also able to reduce infarct size associated with coronary artery ligation, an effect that was also dependent on CB_2 signalling through ERK1/2 pathways (Montecucco *et al.*, 2009). Interestingly, Montecucco *et al.*, (2009) reported that CB_2 receptor mRNA was reduced in hearts that had suffered ischaemia compared to sham operated controls. *In vivo,* the phytocannabinoid CBD also shows cardio-protective qualities (Durst *et al.*, 2007). CBD when given 1 hour before coronary artery ligation reduced infarct size and promoted increased left ventricular ejection fraction that were associated with decreased inflammation (Durst *et al.*, 2007). A further study also reported that CBD given prior to coronary artery occlusion produced reductions in infarction and also inhibited arrhythmia associated with infarction (Walsh *et al.*, 2010). CBD also inhibits cardiodysfunction and cardiomyocyte damage induced by a high

glucose environment (Rajesh *et al.,* 2010). In streptozotocin-induced diabetic mice, diabetes was associated with impaired diastolic and systolic left ventricular function that was restored by CBD pre-treatment (Rajesh *et al.,* 2010). In this model, the effects of CBD were associated with decreased inflammatory mediators, decreased oxidative stress, decreased nitrative stress and attenuation of high glucose-induced apoptosis (Rajesh *et al.,* 2010). It has recently been shown by the same group, using the same model of diabetes, that genetic deletion of the CB_1 receptor reduces the oxidative/nitrative stress, inflammation and fibrosis associated with chronic cardiomyopathy caused by diabetic diabetes (Rajesh *et al.,* 2012).

In humans with heart disease, there is a consensus that endocannabinoid levels are raised when compared to healthy controls (Sugamura *et al.,* 2009; Weis *et al.,* 2010). However, receptor expression differs and this is presumably dependent on the specific type of heart disease. In the Sugamura *et al.* (2009) study it was reported that, in patients with unstable angina coronary artery plaque, content of CB_1 receptors is increased when compared to patients with stable angina. In this study the level of the CB_2 receptor was reported to be lower than that of CB_1 expression, however CB_2 receptor expression is not compared to patients with stable angina. The Weis *et al.* (2010) study reported that CB_1 and CB_2 receptor protein expression was equal in autopsy samples of left ventricular myocardium taken from individuals who had died with no known heart condition. However, in chronic heart failure patients, CB_1 protein expression was reduced, whereas CB_2 receptor protein expression was elevated and mRNA expression was elevated 11 fold (Weis *et al.,* 2010).

Hypertension

In models of hypertension the effects of cannabinoids have been shown to have different effects depending on the model of hypertension used and the experimental conditions.

In anaesthetized rats, injection of AEA causes a tri-phasic haemodynamic response initially causing hypotension followed by a brief pressor effect and then by a prolonged hypotension (Lake *et al.,* 1997). However, in spontaneously hypertensive rats (SHR) AEA causes a greater hypotensive response (Lake *et al.,* 1997). A later study reported that in anaesthetised Wistar Kyoto (WKY) rats injection with

AM251 had no effect on blood pressure, yet when injected into three different types of hypertensive model (SHR, Dahl salt-sensitive and angiotensin II-induced hypertensive Sprague-Dawley rats) it significantly increased blood pressure (Batkai *et al.*, 2004). A further finding of interest was that injection with URB597 decreased blood pressure in hypertensive rat models, an effect that could be inhibited using AM251 (Batkai *et al.*, 2004). Immunochemical analysis of the hypertensive rats used in this study revealed increased CB_1 receptor expression, increased FAAH expression, decreased AEA levels and unaltered 2-AG levels (Batkai *et al.*, 2004).

In conscious freely-moving rats, CB_1 antagonism has a similar hypertensive effect in SHR rats that is not seen in WKY controls (Wheal *et al.*, 2007). In this same model it was shown that infusion with AEA caused a rise followed by a fall in blood pressure which was not associated with vasorelaxation of the mesenteric or hindquarters beds (Wheal *et al.*, 2007). WIN55,212-2 was also used in this study, and the findings were the same as those for AEA albeit slightly more pronounced (Wheal *et al.*, 2007). The responses to both AEA and WIN55,212-2 were not mediated by the CB_1 receptor and the overall responses were considered to be detrimental to cardiac function (Wheal *et al.*, 2007). However, in models of acute hypertension using Sprague-Dawley rats made hypertensive using angiotensin II, both AEA and WIN55,212-2 were able to cause hypotension which was accompanied by vasodilation in the hindquarters, renal and mesenteric vascular beds (Ho and Gardiner 2009). In this study it was also shown that co-administration of URB597 with AEA enhanced the hypotensive effects of AEA, and that the responses to WIN55,212-2 but not AEA were inhibited using AM251 (Ho and Gardiner 2009).

In isolated arteries the vasorelaxant effects of AEA are less potent in the perfused whole mesenteric vascular beds of SHR rats than in WKY controls (Wheal and Randall 2009). Capsaicin pre-treatment reduced the effects of AEA in both vascular beds, however the potency of AEA still remained enhanced in WKY compared to SHR rats (Wheal and Randall 2009). Pre-treatment with L-NAME however significantly reduced the potency of AEA in WKY in so much that there was no difference between WKY and SHR in responses to AEA (Wheal and Randall 2009). The same study then went on to the look at the effects of AEA in aortic ring segments from SHR and WKY rats. This showed

that the maximal response of AEA was enhanced in SHR aortic rings in a manner that was endothelium but not NO-dependent (Wheal and Randall 2009). Earlier work looking at the effects of THC reported that, in rats made hypertensive using L-NAME, THC-induced vasorelaxation was significantly enhanced in the mesenteric resistance arteries (O'Sullivan *et al.,* 2007). In both L-NAME-treated and control arteries the effects of THC were reduced, with a greater reduction observed in the arteries from L-NAME-treated rats (O'Sullivan *et al.,* 2007). However, in L-NAME-treated arteries vasorelaxation was also dependent on COX, a finding not observed in control arteries (O'Sullivan *et al.,* 2007). Recent research has also shown a COX-dependent enhancement of vasorelaxation induced by oleamide in aortic rings of SHR rats compared to WKY rats (Hopps *et al.,* 2012).

Taken together these studies show that in unconscious animal studies and isolated tissue studies the vasorelaxant effects of cannabinoids are enhanced in models of hypertension. The enhanced effects seen are more commonly associated with the CB_1 receptor modulation of sympathetic tone in *in vivo* studies while in isolated tissues pathways vary depending on the cannabinoid ligand. However, in conscious-freely moving animals the effects of cannabinoids in chronically hypertensive animals have been considered detrimental to cardiac function as the hypotension observed is not associated with vascular bed vasodilation. In acutely hypertensive freely-moving rats however, cannabinoids have been associated with enhanced hypotension and vasorelaxation of vascular beds suggesting a positive role in the cardiovascular system.

1.3. The Effects of Cannabinoids in the Human Vasculature

1.3.1. The Exogenous Effects of Cannabinoids *In Vivo*

In vivo research on the hypotensive effects of cannabinoids revealed hypotension in some participants but not others. Intrapulmonary administration of pure THC using a vaporizer did not affect blood pressure (Zuurman *et al.,* 2008). Also, smoking marijuana cigarettes caused inconsistent effects on blood pressure, however symptomatic hypotension was reported in 22% of participants (Gorelick *et al.,* 2006). Interestingly, when participants were given rimonabant alone there were no alterations in blood pressure, and in the marijuana

cigarette group symptomatic hypotension was no longer reported (Gorelick *et al.,* 2006). A further study on the effects of marijuana cigarettes has shown that heart rate was increased to >140 bpm in 50% of participants and 25% of participants experienced hypotension which lasted around 45 minutes (Hunault *et al.,* 2008). In human forearm microcirculation however, interarterial injection of AEA had no effect on vascular tone, whilst topical application caused vasodilation in a TRPV1 mediated manner (Movahed *et al.,* 2005).

1.3.2. Research *in vitro*

The first published *in vitro* report in human arteries showed that AEA and methanandamide (at concentrations up to 30 μM) were without vasorelaxant effects in myometrial arteries taken from healthy patients without reports of hypertensive pregnancies (Kenny *et al.,* 2002). In isolated human pulmonary arteries, virodhamine, Abn-CBD and anandamide have been shown to cause vasorelaxation (Kozlowska *et al.,* 2007; Kozlowska *et al.,* 2008). These relaxations were endothelium-dependent and insensitive to CB_1 antagonism. Antagonism of the CB_e receptor, using O-1918 (10 μM) or CBD (3 μM), reduced the potency of both Abn-CBD and virodhamine. Furthermore, virodhamine potency was also reduced in the presence of the potassium channel blockers charybdotoxin and apamin (Kozlowska *et al.,* 2007; Kozlowska *et al.,* 2008). The effects of pertussis toxin completely abolished the effects of Abn-CBD (Kozlowska *et al.,* 2007).

1.4. Summary

This review has highlighted the endocannabinoid system and discussed receptor target sites for both endocannabinoids and phytocannabinoids. This review has also shown that many cannabinoid ligands cause vasorelaxation in the vasculature of animals through well-established pathways. It is apparent from this review that the effects of cannabinoids are often dependent on the cannabinoid ligand, the species studied and the vascular bed used. To date, it has been shown that endocannabinoids and cannabinoid receptors are expressed within the human vasculature yet only four studies have been carried out to establish the pharmacological effects of cannabinoids in human arteries. These works have already revealed some discrepancies

between human and animal work. Therefore there is a need for further human work to be carried out to understand the effects of different cannabinoid ligands in differing vascular beds.

In rat and bovine arteries only, cannabinoids have been shown to induce time-dependent vasorelaxation that can be inhibited through antagonism of nuclear receptors. Furthermore, incubation with cannabinoids has been shown to enhance endothelium-dependent vasorelaxation through cannabinoid interactions with nuclear receptors, suggesting a possible therapeutic for disease states associated with a decline in endothelium-dependent vasorelaxation. However, time-dependent, nuclear receptor-mediated vasorelaxation has not been investigated in human arteries. Given the potential therapeutic scope for this cannabinoid-induced effect there is a definite need to conduct work to see if this effect carries over in humans.

Finally the effects of cannabinoids are altered in models of disease, with cannabinoid-induced vasorelaxation/hypotension being enhanced in some states. Similarly, current human work has shown that in disease states endocannabinoid levels are often raised, yet the effects of cannabinoids in isolated arteries from humans with vascular disease are unknown.

1.5. Aims

The first aim of the present study was to screen a range of cannabinoid compounds for their ability to induce vasorelaxation in human mesenteric arteries. The second aim of this study was to probe the mechanisms of action of the most efficacious endocannabinoid and phytocannabinoid. The third aim of this study was then to investigate a potential role for PPARγ-mediated time-dependent vasorelaxation of cannabinoids in human mesenteric arteries. Finally all cannabinoid responses were assessed in relation to the medical condition from the patient that they were taken.

2. MATERIALS AND METHODS

2.1. Chemicals, Buffers and Drug solutions

2.1.1. General Chemicals

Table 2.1 shows the salt composition of buffers used in this study. Table 2.2 shows the chemicals that were used in this study. Purchasing, stock concentration, storage and bath concentration are all given in the relevant columns.

Table 2.1. Composition of buffers used.
Composition of buffers used in this study, buffers were made as required and all dilutions were made in triple distilled water

Buffer	Salt	Purchased	Concentration mM/L
PSS	Sodium chloride (NaCl)	Sigma	119
	Potassium chloride (KCl)	Sigma	4.7
	Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Sigma	2.5
	Magnesium sulphate ($MgSO_4.7H_2O$)	Sigma	1.17
	Sodium bicarbonate ($NaHCO_3$)	Sigma	25
	Potassium dihydrogen phosphate (KH_2PO_4)	Sigma	1.18
	Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$)	Sigma	0.027
	Glucose ($C_6H_{12}O_6$)	Sigma	5.5
KPSS	As with PSS with the following exceptions		
	Exclusion of NaCl	Sigma	
	Inclusion of KCl	Sigma	123.7
Ca^{2+} free PSS	As with PSS with the exclusion of $CaCl_2.2H_2O$		
Ca^{2+} free KPSS	As with KPSS with the exclusion of $CaCl_2.2H_2O$		

Table 2.2. Chemicals and dilutions.
Chemicals used in this study with stock concentrations and storage. Further dilutions from stock were made in triple distilled water. M.A.R denotes made as required.

Chemical	Purchased	Stock concentration	Storage	Bath concentration
2-AG	Ascent	10 mM in EtOH	-20°C	0.1-100 μM
Acetylcholine	Sigma	10 mM H_20	M.A.R	10 μM
AH6809	Sigma	10 mM in EtOH	-20°C	1 & 10 μM
AM251	Tocris	10 mM in DMSO	-20°C	100 nM
AM630	Tocris	10 mM in DMSO	-20°C	100 nM
Anandamide	Tocris	10 mM in EtOH	-20°C	0.1-100 μM
Bradykinin	Sigma	10 mM H_20	-20°C	10 μM
Cannabidiol	GW Pharma	10 mM in EtOH	-20°C	0.1-100 μM
Capsaicin	Tocris	10 mM in EtOH	M.A.R	10 μM
CAY10441	Cayman	10 mM in DMSO	-20°C	100 nM
CP55,940	Tocris	10 mM in EtOH	-20°C	0.1-100 μM
Endothelin-1	Sigma	0.01 mM in H_20	-20°C	1-10 nM
Flurbiprofen	Tocris	10 μM in PSS	M.A.R	10 μM
GW9662	Sigma	10 mM in EtOH	-20°C	1 μM
HU308	Tocris	10 mM in EtOH	-20°C	0.1-100 μM
Indomethacin	Sigma	10 μM in PSS	M.A.R	10 μM
JZL 184	Sigma	10 mM in DMSO	-20°C	1 μM
L161982	Tocris	10 mM in DMSO	-20°C	1 μM
L-NAME	Sigma	10 μM in PSS	M.A.R	300 μM
LY320135	Tocris	10 mM in DMSO	-20°C	1 μM
Methoxamine	Sigma	10 mM in H_20	-20°C	1-50 μM
Nimesulide	Tocris	10 μM in PSS	M.A.R	10 μM
Noradrenaline	Sigma	0.1 mM in H_20	-20°C	0.1-1 μM
O-1918	Tocris	10 mM in DMSO	-20°C	10 μM
THC	Tocris	10 mM in EtOH	-20°C	0.1-100 μM
U46619	Tocris	0.1 mM in H_20	-20°C	50-300 nM
URB597	Sigma	10 mM in DMSO	-20°C	1 μM

2.2. Ethics and Tissue Acquisition

2.2.1. Ethical Considerations

This study received ethical approval from the Derbyshire Research Ethics Committee (REC) and Derby Hospitals Foundation Trust Research and Development. It was carried out in accordance with the ethical principles outlined in the declaration of Helsinki 1996. Other ethical influences on this project have been the International Conference on Harmonisation-Guideline for Good Clinical Practice (sections 4 and 5), the Department of Health Governance Framework for health and social care 2nd edition 2005 (sections 2 and 3), the Human Tissue Act 2004 chapter 30 and the Data Protection Act 1998.

2.2.2. Patient Inclusion and Consent

Patients undergoing colorectal resections were identified from the elective theatre list. Patients were included in this study if they were above sixteen years of age and undergoing colorectal re-section for invasive carcinoma, colorectal polyps, Crohn's disease or diverticular disease. Patients were excluded from this study if they were undergoing emergency surgery, unable to give informed consent, had a mental incapacity to give informed consent or patients who did not understand written or verbal information in English. Appropriate patients were given a Patient Information Sheet in the outpatient clinic several days/weeks before surgery. On the morning of the surgery the study was discussed with the patient and any questions were answered, written informed consent was then obtained. A Unique Patient ID was allocated to all patients on the consent form. This ensured that the patient remained anonymous. All patient information including consent forms and computer files containing patient medical notes were kept in locked cabinets or password protected. Access to such files was limited to staff working on the study.

2.2.3. Patient Information

After the patients had given informed consent the patient's pre-operative assessment form was viewed. This provided information containing the patient's medical history and a list of current medication, of particular interest was any conditions/medications with a known impact on vascular function. The information taken was used to fill out the patient information sheet, and used for *post-hoc* analysis. Patient

information sheets were made anonymous and stored as stated in section 2.2.2.

2.2.4. Tissue Acquisition and Transportation

A 2-3 cm square piece of mesenteric tissue was dissected from the colon by a histopathologist and placed in physiological salt solution (PSS, see table 1 for composition), within 1 hour of removal from the patient. Tissue was placed on ice and transported back to the laboratory. Tissue was dissected into arterial segments and stored at 4°C in fresh PSS solution until analysis. The time between tissue storage and analysis was always within 24 hours. However on average, tissue was used within 10 hours and the maximal time between tissue collection and analysis was 22 hours.

2.3. Dissecting and Mounting

2.3.1. Dissection Procedure

Mesenteric arteries were dissected from tissue samples under a microscope using fine forceps and scissors (World Precision Instruments, Hertfordshire, England). Arteries were identified from veins due to their thicker muscular walls and an acute angle at the site of branching (Figure 2.1A). The luminal diameter of the artery was estimated according to the chosen experiment (<1 mm, for wire myography; >1 mm, for fixed hook myography). Appropriate arteries were identified, carefully dissected from mesenteric tissue and cleaned of all remaining connective and adipose tissue. Arteries were cut into 2 mm segments and placed in fresh PSS solution at 4°C and stored until use. However, on average the arteries were used immediately and therefore were not stored. The maximum length of storage was 15 hours.

2.3.2. Myography General

Myographs were cleaned daily and calibrated on a bi-monthly basis as per the manufacturer's guidelines. Myography was carried out on a DMT myograph (DMT 620 myograph system, Aarhus, Denmark), using either wire or fixed hook myograph fixings. Recordings were made using lab chart5 (ADInstruments, Oxford, England). Myograph chambers were filled with 5 mL fluid, kept at 37°C and gassed with 5% CO_2 in O_2.

2.3.3. Wire Myography Mounting Procedure

A 40 µm tungsten wire was secured under a mounting screw with the unsecured end clamped in position by the myograph jaws. A 2 mm arterial segment was carefully positioned at the end of the wire, the artery was pulled over the wire and into the gap between the myograph jaws. The remaining free end of the wire was secured. A second piece of wire was passed through the artery and secured to the screws on the opposite myograph jaw. The mount was assessed to ensure wires were tight, parallel and that the artery sat in the middle of the jaws free of tension or obstruction.

2.3.4. Fixed Hook Myography Mounting Procedure

Fixed hooks were assessed to ensure they were parallel with each other. A 2 mm long arterial segment was placed in the myograph chamber. The segment was lightly manipulated and mounted over both fixed hooks. Once on the hooks the artery rested under no tension.

2.3.5. Normalisation

Arteries were normalised, setting each artery at an internal diameter that produced 90% of the tension which would occur at 13.3 kPa (Lab chart five normalisation software, ADInstruments), this process involved; arterial tension being increased in a stepwise manner using the micrometer screw to open the myograph jaws/fixed hooks. On turning the micrometer arterial tension would sharply increase, and gradually relax to a plateau above baseline over a period of 2 minutes (Figure 2.1B). This was repeated a minimum of four times until the target force was achieved (Figure 2.1B). The force and micrometer readings from this process were used to determine arterial wall tension (mN/mm) and internal circumference (µm). These values were used to form an exponential curve, which was fitted to an isobar curve corresponding to 13.3 kPa. The tension and internal circumference that occur at 13.3 kPa (IC_{100}) is determined by the point at which the exponential curve intersects the isobar curve, which is determined using the Laplace equation (Mulvany and Halpern 1977). The artery was set to a baseline tension that produced 90% of IC_{100} which enables rat mesenteric arteries to generate optimal active tension (Mulvany and Halpern 1977). In human arteries this is also more effective than other methods available for generating optimal contractile responses from tissues (Wareing *et al.,* 2002). Computational and mathematical

modelling have suggested that the human mesenteric circulation is typically exposed to between 10.2 kPa and 14.6 kPa of pressure (Mabotuwana *et al.,* 2007).

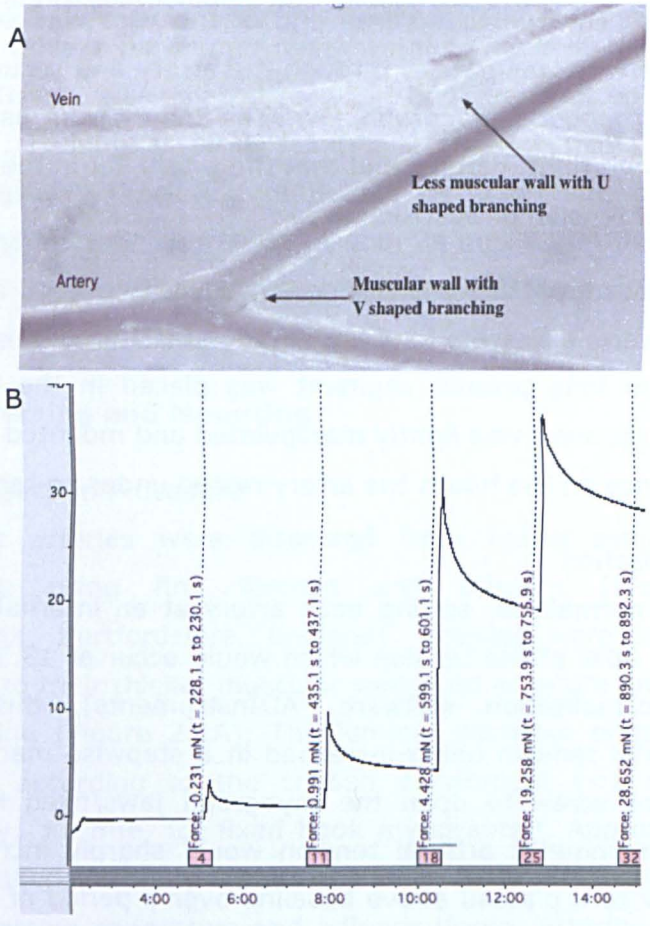

Figure 2.1 Artery dissection and normalisation.
Difference between arteries and veins can be seen (A). Increase and decrease in arterial force during normalisation (B). Force generated in (B) is then fitted to an isobar curve using the Laplace equation to determine arterial circumference at 90% 100 mmHg.

2.4. Preliminary Studies

2.4.1. Testing Contractile Agents on Human Mesenteric Arteries

In vitro human mesenteric arteries were contract by depolarisation using 124 mM high potassium physiological saline solution (Tottrup and Kraglund 2004), or in the presence of the receptor agonists; noradrenaline (non-selective α/β-adrenoceptor agonist (Romero *et al.,* 1997; Vaughan *et al.,* 2005)), U46619 (TP receptor agonist (Tottrup and Kraglund 2004; Hall *et al.,* 2006)), endothelin-1 (ET_A and ET_B

receptor agonist (Ferrero *et al.*, 2008)) and methoxamine (selective α_1-agonist (Vaughan *et al.*, 2005)). Preliminary experiments were conducted to establish suitable agents and concentrations for contraction. In these experiments arteries were mounted and normalised as above. Once arteries were equilibrated and had stable tone, contractile agents were added in various concentrations (with the exception of KPSS, that was always at 124 mM), to achieve a stable contraction greater than 5 mN net contraction. These experiments revealed that noradrenaline and methoxamine were both unable to give stable or reliable contractions (Figure 2.2A & B). U46619 and endothelin-1 given alone produced reliable contractions that were similar to KPSS responses. However, higher doses of contractile agent were needed and arterial stability was often compromised (Figure 2.2C). Given together, at lower concentrations, U46619 and endothelin-1 produced stable contractions that were comparable to KPSS responses (Figure 2.3A and B). Therefore in further experiments combinations of U46619 and endothelin-1 were used.

2.4.2. Testing Vasorelaxant Agents on Human Mesenteric Arteries

In vitro, in human mesenteric arteries, endothelium-dependent vasorelaxation is observed in the presence of acetylcholine (ACh) (Hutri-Kahonen *et al.*, 1999) and bradykinin (BK) (Ferrero *et al.*, 2008). Preliminary experiments were therefore conducted to establish an appropriate agent and concentration to show the presence of a functioning endothelium. In these experiments arterial segments were contracted using combinations of U46619 and endothelin-1. Once a stable contraction above 5 mN was achieved, a single dose (10 μM) of ACh or BK was added to the myograph chamber. ACh caused vasorelaxation (37 ± 6% [mean ± standard error of mean (s.e.m)] n=7) (Figure 2.3B), which is lower than has previously been reported (Hutri-Kahonen *et al.*, 1999). However BK caused vasorelaxation (74 ± 6%, n=7) (Figure 2.3A) similar to that previously reported (Ferrero *et al.*, 2008). Therefore, vasorelaxation to bradykinin that was ≥70% was used to show the presence of a functioning endothelium.

2.4.3. Standard Start Protocol

A standard start protocol was then devised in order to control for variation in arterial function. The protocol was based on experiments carried out in 2.4.1 and 2.4.2 and was designed to test contractile

ability and endothelium-dependent vasorelaxation. Arterial contraction to KPSS is mediated via membrane depolarisation and calcium influx through voltage-operated calcium channels (Karaki *et al.,* 1997). Arterial contraction to U46619 is mediated through TP receptor activation and subsequent release of calcium from intracellular stores (Hall *et al.,* 2006). Therefore, testing artery contractions in such a way enabled both these pathways to be observed. BK-induced vasorelaxation is endothelium-dependent and associated with NO production and potassium channel activation (Chadha *et al.,* 2011). Therefore, testing arteries in this way allowed for the assessment of the function of the endothelium. To carry out these assessments, arteries were dissected, mounted and normalised as section 2.3. Once arterial segments were normalised 124 mM KPSS was added to the myograph chamber, if a contraction of less than 5 mN was observed the artery was discarded. In arteries where the contractile response was greater than 5 nM the chamber was washed out a minimum of three times with PSS. Once baseline arterial tone had stabilised, 50 nM U46619 was added to the myograph chamber. If a contraction greater than 5 mN was not observed, the artery was discarded. However, in arteries where a stable contraction greater than 5 mN was observed a concentration of 10 μM BK was added. Arteries were discarded if a vasorelaxation less than 70% was achieved. Arteries were then washed out a minimum of three times and arterial tone allowed to stabilise before conducting cannabinoid experiments. This protocol can be viewed as a schematic in Figure 2.4.

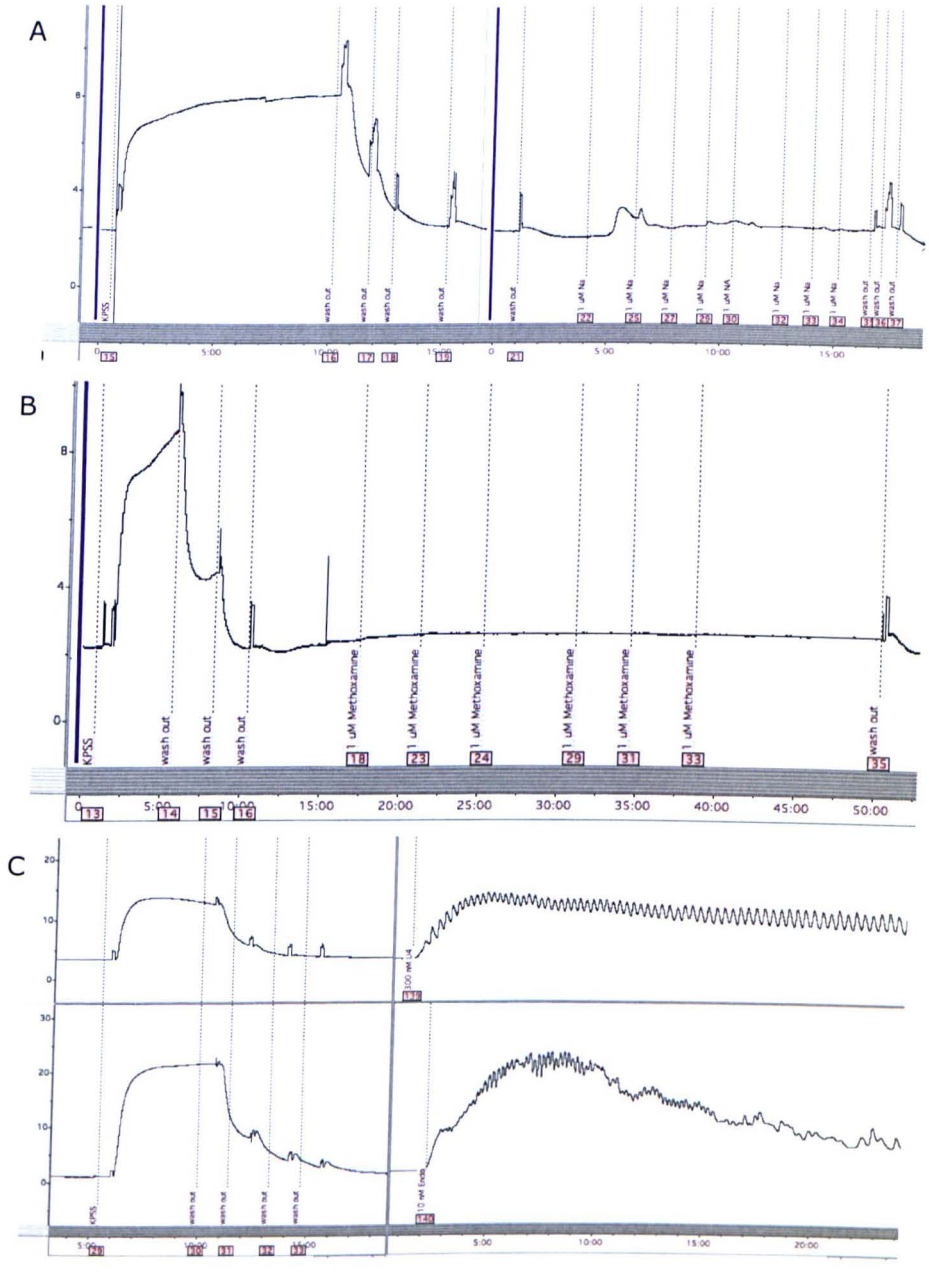

Figure 2.2 Preliminary studies trace data.
Trace data showing the effects of the contractile agents, noradrenaline (A), methoxamine (B), endothelin-1 (top pane C) and U46619 (lower pane C).

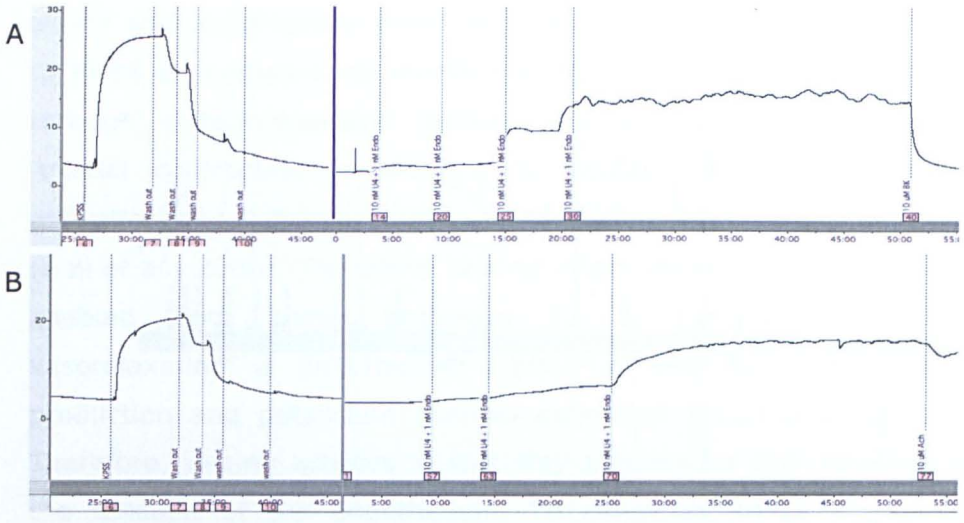

Figure 2.3 Endothelium-dependent vasorelaxant agents.
Screening endothelium-dependent vasorelaxation to BK (A) and ACh (B) in arteries contracted using U46619 and endothelin-1 in combination.

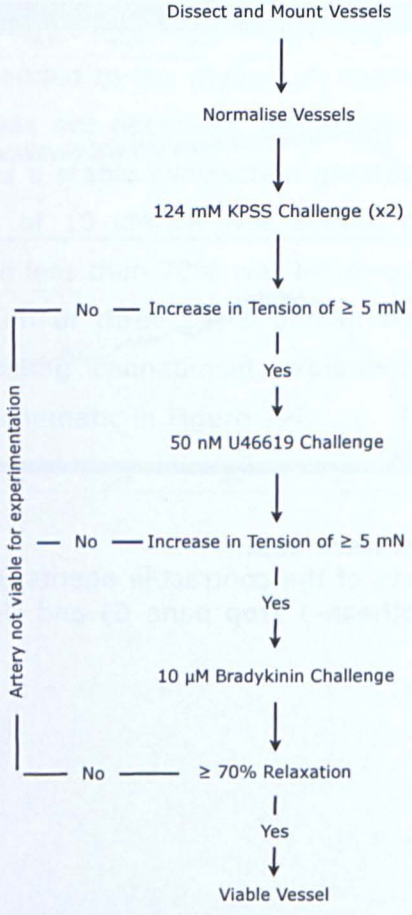

Figure 2.4 Standard start protocol
Protocol derived from preliminary experiments and was subsequently carried out to validate arterial segments.

2.5. Concentration-Response Curves

2.5.1. Contraction and Artery Stability

Viable arteries were washed out a minimum of three times with fresh PSS and baseline allowed to stabilise over ≈20 minutes. Arteries were exposed to 50 nM U46619 and 1 nM endothlin-1 to induce contraction. If an increase of 5 mN force was not achieved, further U46619 and endothlin-1 was added until a maximum of 300 nM U46619 or 10 nM endothelin-1 had been added. Contractions were allowed to stabilise over a period of ≈20 minutes Figure 2.5A.

2.5.2. Addition of Cannabinoid

Concentrations of cannabinoids were added in five minute intervals in a cumulative manner (O'Sullivan *et al.*, 2004). Cannabinoids and vehicle were diluted as described in table 2.3. Once a stable contraction had been achieved, cannabinoid (100 nM-100 μM) or ethanol (0.001-1%) was added to the chamber as described in table 2.3. Figure 2.5A shows a trace sample of a concentration-response curve.

Table 2.3 Concentration-response curve
Volume and concentrations used in concentration-response curves in all cannabinoid experiments. Additions were made to the myograph chamber in 5-minute intervals.

Compound	Stock Concentration	Volume Added to Bath	Cumulative Bath Concentration
Ethanol Vehicle	1% in H_2O	5 µL	0.001%
	1% in H_2O	10 µL	0.003%
	1% in H_2O	35 µL	0.01%
	10% in H_2O	10 µL	0.03%
	10% in H_2O	35 µL	0.1%
	10% in H_2O	100 µL	0.3%
	100% in H_2O	35 µL	1%
Cannabinoid	100 µM in H_2O	5 µL	100 nM
	100 µM in H_2O	10 µL	300 nM
	100 µM in H_2O	35 µL	1 µM
	1 mM in H_2O	10 µL	3 µM
	1 mM in H_2O	35 µL	10 µM
	1 mM in H_2O	100 µL	30 µM
	10 mM in EtOH	35 µL	100 µM

2.5.3. Measurements

Measurements of artery responses were taken in the final minute after cannabinoid addition/vehicle addition (Figure 2.5A). Responses were determined as shown in equation 1.

$$\%\ reduction\ in\ pre-imposed\ tone = (Contractile\ tone - drug\ response) \div (Contractile\ tone - baseline\ tone) \times 100$$

Equation 1. Equation used to calculate the %reduction in pre-imposed tone

2.5.4. Time-dependence Experiments

To establish potential time-dependent vasorelaxation induced by cannabinoids, the largest arteries available in mesenteric tissue samples (>1 mm) were dissected. Arteries were mounted onto fixed hooks and subjected to the same standard start protocol as previously described. Viable arteries were contracted using a combination of

U46619 and endothelin-1 and allowed to stabilise. Once vessel stability had been maintained for ≈20 minutes, a single concentration of cannabinoid or vehicle control was added. Measurements were taken directly before addition of cannabinoid or vehicle and at 15 minute intervals thereafter (O'Sullivan *et al.*, 2005) (Figure 2.5B)

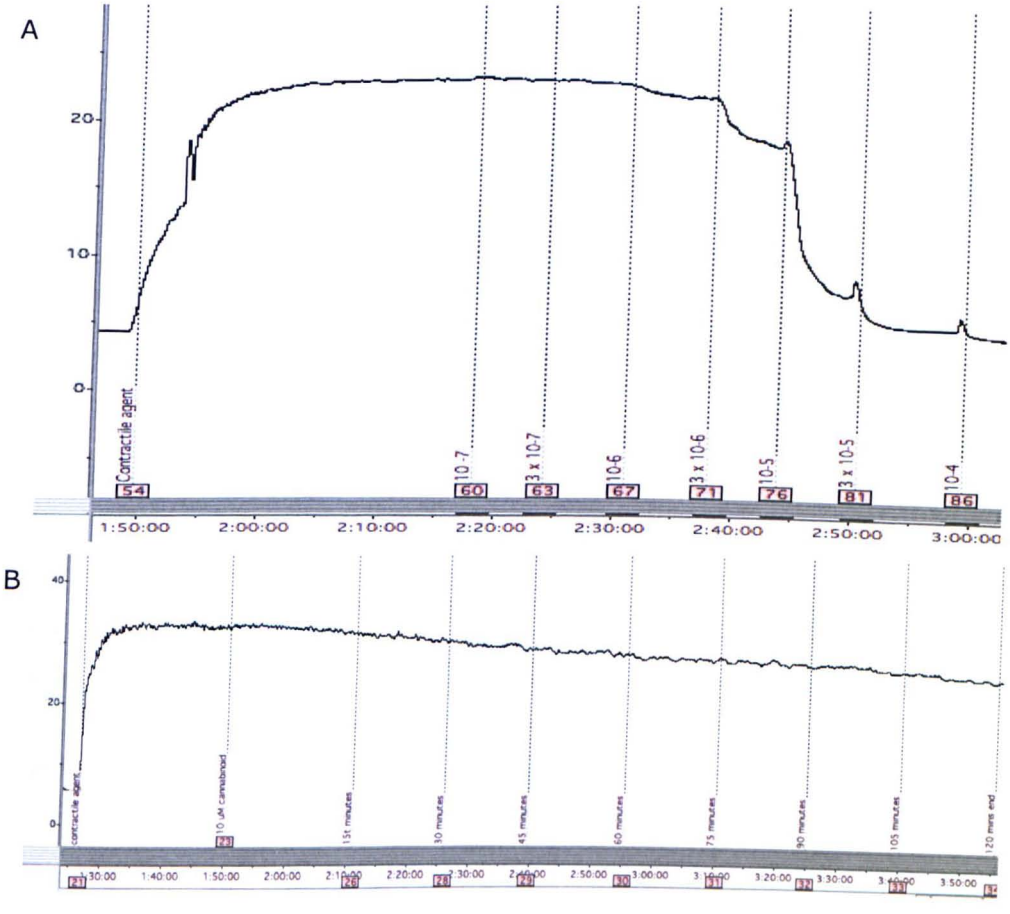

Figure 2.5. Cannabinoid responses.
Traces of typical concentration response curve (A), or time-dependent response curve (B). Cannabinoids were added, either in 5-minute intervals (A) or as one concentration (B), once a stable contraction had been maintained for 20 minutes. Responses were taken in the last minute of each 5-minute period for acute vasorelaxation studies (A), or in the last minute of each 15-minute period in time dependence studies (B).

2.6. Tissue Overnight Storage

2.6.1. Storage Procedure

For feasibility reasons some tissues were stored overnight (4°C) and used the following day. All arterial segments stored overnight were used within 24 hours of collection from theatre.

2.6.2. Assessment of Contractile Responses in Arterial Segments Used Fresh and After Storage

To assess the effects of overnight storage, the second KPSS and the 50 nM U46619 responses were compared between fresh arteries and arteries that had been stored overnight. The average second contraction to KPSS was not significantly different between arteries used fresh or arteries stored overnight (fresh artery segments 23 ± 1 mN, n=291; arterial segments stored overnight 21 ± 1 mN, n=197; see Figure 2.6A). The second KPSS response was taken as the arteries maximum contractile response, therefore the response to 50 nM U46619 was expressed as a percentage of the second KPSS. Responses to 50 nM U46619 were also unaltered by overnight storage of arterial segments (fresh artery segments 85 ± 2% 2nd KPSS [mean ± s.e.m], n=291; artery stored overnight 88 ± 2% 2nd KPSS, n=197; see Figure 2.6B).

2.6.3. Assessment of Bradykinin in Arteries Used Fresh and After Storage

To assess whether overnight storage affects endothelium-dependent vasorelaxation, 10 μM bradykinin responses were compared between both sets of arteries. Bradykinin responses were not significantly altered by overnight storage of arteries (fresh artery segments 88 ± 1% relaxation, n=258; artery segments stored overnight 85 ± 1% relaxation, n=181; see Figure 2.6C)

2.6.4. Assessment of Cannabinoid Responses in Arteries Used Fresh and After Storage

To assess whether overnight storage affects concentration-response curves to cannabinoids comparisons were made between both sets of arteries (n=6). There was no difference between cannabinoid vasorelaxant responses in arteries used fresh compared to those stored overnight (Figure 2.7A, B, C, D & E). Given that contractile and vasorelaxant responses (cannabinoid and bradykinin) were unaltered, subsequent studies were carried out either fresh (66% of patient samples) or after overnight storage (34% of patient samples).

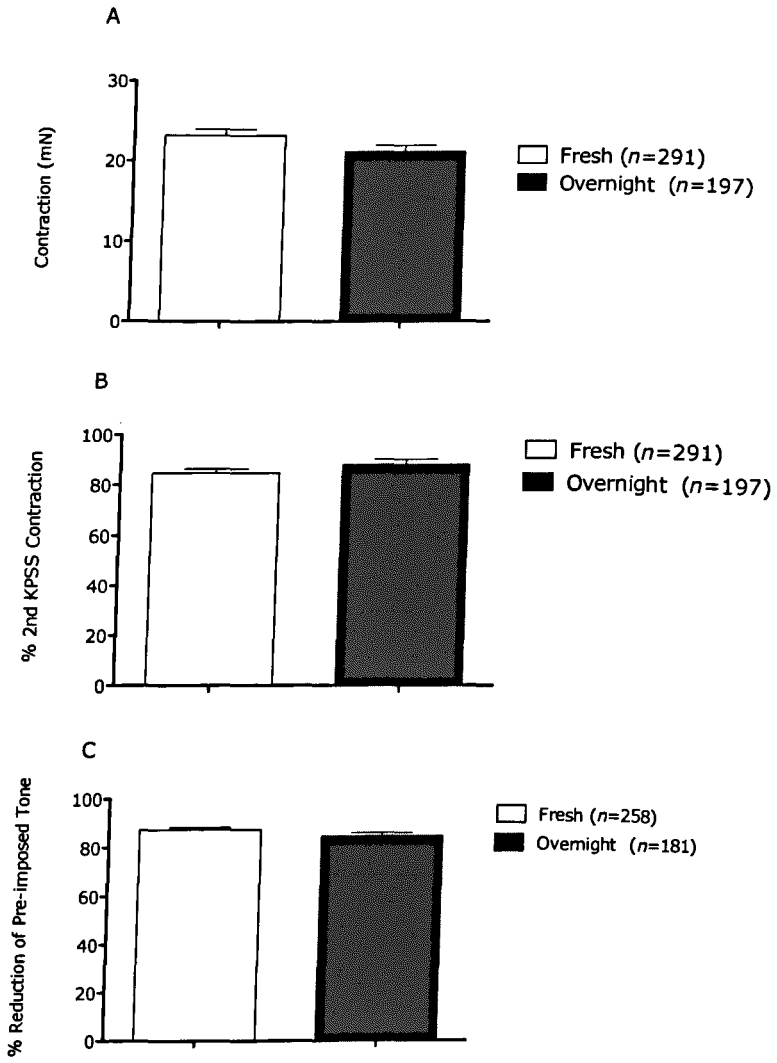

Figure 2.6 Effects of overnight storage (non-cannabinoid responses) Comparisons of vascular responses to 124 mM KPSS (A), 50 nM U46619 (B) and 10 μM bradykinin (C) in arteries used fresh or after overnight storage. Bars represent mean with error bars representing s.e.m. Comparisons were made using Mann Whitney test with $P<0.05$ taken as significant.

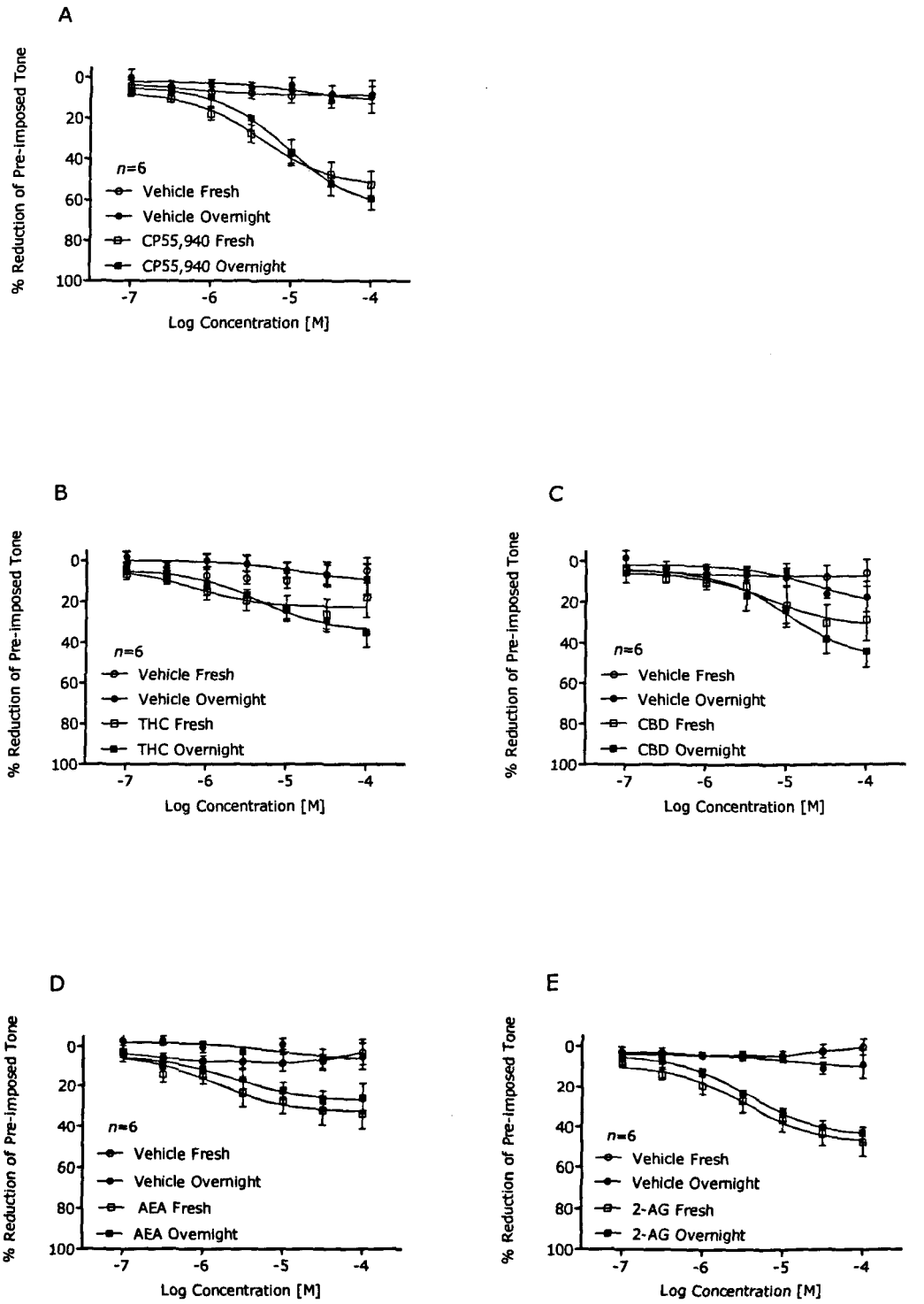

Figure 2.7 Effects of overnight storage (cannabinoid responses) Comparisons between vasorelaxation responses to CP55,940 (A), CBD (B), THC (C), 2-AG (D) and AEA (E) in arteries used fresh or after overnight storage. Data points represent means with error bars representing s.e.m. Comparisons made between arteries used fresh and after overnight storage using 2-way analysis of variance of concentration response curve with $P<0.05$ taken as significant.

2.7. Pharmacological Investigations

2.7.1. Receptor Antagonism

Cannabinoids act through a range of receptors in the vasculature (reviewed Randall *et al.,* 2004). To probe the potential receptor involvement underpinning the vasorelaxant responses to cannabinoids a range of antagonists was used.

AM251

AM251 is an analogue of rimonabant, synthesised with chlorine substituted for iodine by the Makriyannis group in 1996 (Lan *et al.,* 1996; Gatley *et al.,* 1997). AM251 binds to the CB_1 receptor with a K_i value of 7.49 nM and to the CB_2 receptor with a K_i 2,290 nM (Lan *et al.,* 1999). At high concentrations, it has been suggested that AM251 may act as an inverse agonist or as an allosteric modulator at the CB_1 receptor site (Pertwee 2005). AM251 has also been shown to be an agonist of the novel G protein-coupled receptor GPR55 (Ryberg *et al.,* 2007). AM251 (100 nM) inhibits cannabinoid-induced vasorelaxation in a range of vascular preparations (O'Sullivan *et al.,* 2004). AM251 (100 nM (O'Sullivan *et al.,* 2004) was added to the myograph chamber 10 minutes before arteries were contracted. Incubation with AM251 had no effect on arteries baseline or contractile tone (see tables 2.4, 2.5, 2.6).

LY320135

LY320135 is a benzofuran that is structurally distinct from the pyrazole structures of rimonabant and AM251 and was synthesised by Eli Lilly. LY320135 has affinity for CB_1 of 224 nM and >10,000 nM for the CB_2 receptor (Felder *et al.,* 1998). However, at concentrations of >2 μM LY320135 has been shown to antagonise; muscarinic, $5HT_2$, H_1, adrenoceptors (α_1/α_2) and dopamine (D_1/D_2) receptors (Felder *et al.,* 1998). LY320135 (1 μM) has been shown to reverse the effects of CP55,940 in the rat mesenteric bed (Duncan *et al.,* 2004). LY320135 (1 μM, Duncan *et al.,* 2004) was added to arteries 30 minutes before contraction and did not affect baseline or contractile tone (see table 2.5).

AM630

AM630, an aminoalkylindole synthesised by the Makriyannis group in 1995 (Pertwee *et al.,* 1995), displays affinity for the CB_2 receptor at 31.2 nM and affinity for the CB_1 receptor at 5,152 nM (Ross *et al.,* 1999). AM630 in the micromolar range has been shown to behave as a weak/partial agonist at the CB_1 receptor (Ross *et al.,* 1999). However, it has also been reported that AM630 may act as a CB_1 inverse agonist (Landsman *et al.,* 1998; Vasquez *et al.,* 2003). AM630 has been shown to antagonise the vasorelaxant effects of cannabinoids (McDougall *et al.,* 2008). In this study a concentration of 100 nM was used and, as per (O'Sullivan 2009), this was added to the myograph chamber 10 minutes before contraction. Incubation with AM630 had no effect on vessel baseline or contractile tone (see tables 2.4, 2.5 and 2.6).

O-1918

O-1918 is an analogue of cannabidiol that was synthesised by the Kunos group and is unable to displace [^{3}H] CP55,940 from CB_1 or CB_2 receptors at concentrations up to and including 30,000 nM (Offertaler *et al.,* 2003). O-1918, an antagonist of the proposed novel endothelial cannabinoid receptor, inhibits cannabinoid-induced vasorelaxation in a range of vascular preparations (Offertaler *et al.,* 2003; Hoi and Hiley 2006) including human (Kozlowska *et al.,* 2007; Kozlowska *et al.,* 2008). O-1918 (10 μM, (Kozlowska *et al.,* 2007)) was added ten minutes before contraction. Incubation with O-1918 had no effect on vessel baseline or contractile tone (see tables 2.5 and 2.6).

Capsaicin

Capsaicin is the chemical component responsible for heat and pain associated with chilli peppers. Capsaicin binds to TRPV1 channels with high affinity and can cause desensitisation and closure of the channel (Szallasi and Blumberg 1999). Capsaicin interacts with several targets other than TRPV1 channels including, blockade of K^+ channels, enzyme inhibition and alterations in membrane permeability (Szallasi and Blumberg 1999). In the guinea-pig basilar artery, rat hepatic and rat mesenteric artery pre-treatment with capsaicin (10 μM) inhibits the vasorelaxant response of AEA (Zygmunt *et al.,* 1999). Capsaicin (10 μM, (Zygmunt *et al.,* 1999)) was added to the myograph chamber

and allowed to incubate for 1 hour. Arteries were washed out three times and contracted. Incubation with capsaicin significantly decreased baseline tone when compared to time control but had no effect on contractile tone (see tables 2.4 and 2.6).

Cay10441

Cay10441 is a 2-(phenylamino) imidazoline that demonstrates high affinity binding to human IP receptors with a K_i of around 1.5 nM (Clark *et al.,* 2004). Cay10441 does not bind to prostanoid EP/FP and TP receptors and does not have actions at a range of 30 other receptor and ion channel sites (Clark *et al.,* 2004). However, Cay10441 has been shown to bind to the α_{2A} receptor (K_i 3 μM) and the imidazoline I_2 receptor (K_i 1.5 nM). In rat mesenteric arteries Cay10441 (100 nM) antagonises IP receptor-induced vasorelaxation to novokinin (Yamada *et al.,* 2008). Cay10441 (100 nM, (Yamada *et al.,* 2008)) was added to arteries 10 minutes before contraction. Incubation with Cay10441 had no effect on artery baseline or contractile tone (see table 2.4).

L-161982

L-161982 is a potent antagonist of the prostanoid EP_4 receptor (Machwate *et al.,* 2001). L-161982 also displays affinity at prostanoid EP_1, EP_2, EP_3, FP, IP, DP and TP receptors with K_i values of 19.2, 19.2, 1.9, 5.6, 6.8, 5.1 and 0.7 μM respectively (Machwate *et al.,* 2001). L-161,982, through antagonism of EP_4 receptors, has been shown to inhibit lipo-polysaccharide- (LPS) induced relaxation of mouse trachea (Balzary and Cocks 2006). L-161982 (1 μM (Balzary and Cocks 2006)) was added to the bath 10 minutes before contraction. Incubation with L-161982 had no effect on artery baseline or contractile tone (see table 2.4).

AH6809

AH6809 antagonises prostanoid EP_1, EP_2, EP_3, DP and TP receptors with K_i values of 1.2, 1.2, 1.6, 1.4 and 4.3 μM respectively (Abramovitz *et al.,* 2000). In rat mesenteric arteries, AH6809 (10 μM) inhibits vasorelaxation to 14,15-epoxyeicosatrienoic acid (14,15 EET) through antagonism of the EP_2 receptors (Yang *et al.,* 2010). AH6809 (10 μM (Yang *et al.,* 2010)) was initially added to arteries 30 minutes before contraction. At this concentration AH6809 had no effect on artery

baseline tone but did inhibit contractions (see table 2.4). Therefore a lower concentration of 1 μM was used. This concentration had no effect on artery baseline tone or contractile responses (see table 2.4).

GW9662

In $(AOx)_3$-TK-Luc promoter-transfected RAW264.7 macrophage cells, GW9662 antagonised BRL49653 activation of peroxisome proliferator activated receptor gamma (PPARγ) (Huang *et al.,* 1999). GW9662 binds to PPARγ, PPARα and PPARδ with respective IC_{50} values of 3.3, 32 and 2000 nM (Leesnitzer *et al.,* 2002), and at concentrations of 1 μM has been shown to inhibit the time-dependent vasorelaxant effects of cannabidiol in rat aortae (O'Sullivan *et al.,* 2009). GW9662 (1 μM, (O'Sullivan *et al.,* 2009)) was added to arteries 10 minutes before contraction. Incubation with GW9662 had no effect on baseline tone or contractile response (see table 2.5 and 2.6).

2.7.2. Enzyme Inhibition

Endogenous cannabinoids can be degraded by a variety of different enzymes including fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL) and cyclooxygenase-2 (COX-1/-2) (Muccioli 2010). In some experiments, a range of inhibitors of these enzymes was used.

URB597

The carbamic acid ester URB597 was synthesised by the Piomelli group in 2003 (Kathuria *et al.,* 2003). URB597 has an IC_{50} for fatty acid amide hydrolase of 5.6 nM and has no effect on the expression of monoacylglycerol lipase in rat brain homogenates or the binding of WIN55,212-2 to CB_1 and CB_2 receptors in rat cerebellum membranes or transfected CHO cells respectively (Kathuria *et al.,* 2003). In later studies, it has been shown that URB597 also has no action at a range of other receptors, transporters and enzymes including COX-1/-2 (Piomelli *et al.,* 2006). However, URB 597 (24.5 μM) activates TRPA1 channels in transfected HEK cells (Niforatos *et al.,* 2007). Furthermore, in activity based protein profiling studies using mouse and human liver proteomes URB597, at a concentration range between 0.5-100 μM, showed inhibition of a range of serine hydrolase peptides (Ahn *et al.,* 2007). In the rat mesenteric artery URB597 enhanced the vasorelaxant

responses to anandamide (Ho and Randall 2007). URB597 (1 μM, (Ho and Randall 2007)) was added to arteries 30 minutes before contraction. Incubation with URB597 had no effect on vessel baseline or contractile tone (see table 2.4 and 2.6).

JZL184

JZL184 is a carbamate synthesised as an irreversible inhibitor of monoacylglycerol lipase with an IC_{50} of 8 nM for inhibition of 2-AG hydrolysis (Long *et al.*, 2009). JZL184 also inhibited ABHD6 and FAAH but at a higher concentration (10 μM) (Long *et al.*, 2009). JZL184 (1 μM, (Long *et al.*, 2009)) was added to arteries 30 minutes before contraction. Incubation with JZL184 had no effect on vessel baseline or contractile tone (see table 2.4).

Indomethacin

Indomethacin is a non-steroidal anti-inflammatory drug, which in human blood inhibits both cyclooxygenase isoforms (COX-1 and COX-2) with IC_{50} values of 230 and 630 nM respectively (Palomer *et al.*, 2002). Indomethacin, at 40 and 100 μM, has activities at PPARγ and PPARα respectively (Lehman 1996). At 100 μM, indomethacin inhibits CP55,940 binding to CB_1 receptors, and can inhibit anandamide hydrolysis by fatty acid amide hydrolase (Holt *et al.*, 2007). 10 μM indomethacin caused inhibition of vasorelaxation to virodhamine in the human pulmonary artery (Kozlowska *et al.*, 2008). Indomethacin (10 μM, (Kozlowska *et al.*, 2008)) was added to the arteries 30 minutes before contraction. Incubation with indomethacin had no effect on vessel baseline or contractile tone (see tables 2.4, 2.5 and 2.6).

Flurbiprofen

Flurbiprofen is a non-steroidal anti-inflammatory agent, which preferentially inhibits COX-1 over COX-2 (IC_{50} 100 nM COX-1 compared IC_{50} 400 nM COX-2) (Gierse 1995). Flurbiprofen, at a concentration of 3 μM inhibited capsaicin-induced calcitonin gene-related peptide release, an effect that can be inhibited by AM251 (Seidel *et al.*, 2003), and in a pH dependant manner flurbiprofen inhibits FAAH metabolism of AEA (Fowler *et al.*, 2003). Flurbiprofen (10 μM) potentiates the effects of 2-AG in the rat mesenteric artery (Ho and Randall 2007). Flurbiprofen (10 μM, (Ho and Randall 2007)) was added to arteries 30

minutes before contraction. Incubation with flurbiprofen had no effect on vessel baseline or contractile tone (see table 2.4).

Nimesulide

Nimesulide is a selective COX-2 inhibitor, which binds to COX-2 with an IC_{50} of 10 nM. Nimesulide binds to COX-1 at concentrations greater than 10 μM (Cullen *et al.,* 1998). Unlike flurbiprofen, nimesulide was shown not to affect FAAH metabolism of AEA (Fowler *et al.,* 2003) Nimesulide (10 μM) potentiates the effects of anandamide in the rat mesenteric artery (Ho and Randall 2007). Nimesulide (10 μM, (Ho and Randall 2007)) was added to arteries 30 minutes before contraction. Incubation with nimesulide had no effect on baseline or contractile tone (see table 2.4).

2.7.3. Endothelium Dependence

Cannabinoid-induced vasorelaxation has been shown to have an endothelium-dependent component. This component includes stimulation of nitric oxide release (Deutsch *et al.,* 1997), through activation of an as yet unidentified endothelium-derived hyperpolarising factor (Randall *et al.,* 1996) and activation of an as yet uncharacterised endothelial receptor (Offertaler *et al.,* 2003). In some experiments, techniques were used to test the possibility that cannabinoids responses were endothelium-dependent (O'Sullivan *et al.,* 2004).

L-NAME

L-NAME is an inactive NOS inhibitor that requires hydrolysis to an active form to inhibit NOS (Griffith and Kilbourn 1996). L-NAME inhibits inducible-, neuronal- and endothelial-NOS with an IC_{50} values of 4.5, 6.1 and 5.8 μM respectively (Boer *et al.,* 2000). L-NAME, alone (Deutsch *et al.,* 1997), or in combination with indomethacin and potassium channel blockers (O'Sullivan *et al.,* 2004), has been shown to inhibit endocannabinoid-induced vasorelaxation in a variety of rat vascular beds. L-NAME (300 μM, (O'Sullivan *et al.,* 2004)) was added to arteries after standard start protocol and present throughout. Incubation with L-NAME had no effect on vessel baseline or contractile tone (see tables 2.4, 2.5 and 2.6).

Endothelial Denudation

Vasorelaxation to a range of cannabinoids is reduced via removal of the endothelium (Mukhopadhyay *et al.,* 2002; O'Sullivan *et al.,* 2004; Kozlowska *et al.,* 2007; Kozlowska *et al.,* 2008). In some experiments the endothelium was removed via gentle abrasion with a human forearm hair. The removal of the endothelium was confirmed by <10% vasorelaxation to 10 μM bradykinin. Endothelium denudation had no effect on contractile tone (see tables 2.4, 2.5 and 2.6)

2.7.4. Ion Channel Modulation

In many studies cannabinoids have been shown to modulate ion channel activity either by inhibition of Ca^{2+} channels (Caulfield and Brown 1992; Mackie and Hille 1992; Ross *et al.,* 2008) or through opening of K^+ channels (Hampson *et al.,* 1995). Therefore, in some experiments a potential role for ion channel involvement was assessed. The techniques used are described below with details of the effect these techniques had on vessel tone.

Experiments in the Absence of Ca^{2+}

Calcium flux has a key role in controlling vascular tone; removing extracellular calcium limits the available calcium to that which can be released from intracellular stores. Several vasorelaxant agents including ACh cause release of intracellular calcium and influx of extracellular calcium in their vasorelaxant mechanisms (Socha *et al.,* 2012). To establish a potential role of extracellular calcium, in some arteries, extracellular calcium was removed from the buffer after the standard start protocol. These arteries were incubated for a minimum period of ten minutes, contracted and cannabinoid concentration-response curves were carried out. Removal of Ca^{2+} from PSS had no effect on vessel baseline or contractile tone (see table 2.4).

$CaCl_2$ Contraction Experiments

$CaCl_2$ contractions in high potassium medium are mediated through voltage-operated calcium channels (VOCCs) (Xu *et al.,* 2012). Cannabinoids inhibit a range of VOCCs (Caulfield and Brown 1992; Mackie and Hille 1992; Ross *et al.,* 2008), and oppose $CaCl_2$ contractions in the rat aortae (O'Sullivan *et al.,* 2009). In some arteries the role of Ca^{2+}entry was assessed by bathing arterial segments (10

minutes) in Ca^{2+}-free PSS, then for 10 minutes in Ca^{2+}-free KPSS and then carrying out a concentration-response curve to $CaCl_2$ (0.001-30 mM) in the presence of cannabinoid or vehicle control.

KPSS-contracted arterial segments

KPSS causes depolarisation of arteries and causes contraction through VOCCs (Karaki *et al.,* 1997). Synthetic cannabinoids have previously been shown to relax KPSS-pre-contracted rat mesenteric arteries, through receptor-mediated VOCC inhibition (White and Hiley 1998). KPSS contractions also limit potassium efflux (Quignard *et al.,* 1999). To explore these possibilities, some arteries were contracted with KPSS solution and cannabinoid concentration-responses curves carried out.

Table 2.4 Effects of a range of inhibitors/antagonists on artery baseline tone and contractile responses, 2-AG studies.
The effects of the inhibitors used in 2-AG experiments on baseline tone and contractile responses. Δ alteration in baseline tone determined by the baseline tone after addition of inhibitor/antagonist minus baseline tone before addition of inhibitor/antagonist. Contractile tone to U46619 (10-300 nM) in combination with endothelin-1 (1-10 nM) is expressed as percentage of response to 124 mM KPSS. Values given ± s.e.m with *n* equal to the number of patients. Comparisons made using 1-way ANOVA with Dunnett's *post-hoc* test to compare all responses to time control. ** P<0.01

Artery treatment	Baseline tone mN (Δ alteration)	Contractile Tone (% KPSS)	*n*
Time control	-0.05±0.03	80±4	44
AH6809 (1 μM)	-0.35±0.66	60±7	6
AH6809 (10 μM)	0.9±0.9	34±20**	5
AM251	0.11±0.04	91±13	7
AM630	0.28±0.18	85±13	5
Calcium free PSS	0.31±0.09	73±7	7
Capsaicin	-1.13±0.52**	89±13	6
CAY10441	0.23±0.36	79±6	9
Endothelium-denuded		81±6	8
Flurbiprofen	-0.31±0.27	83±7	6
Indomethacin	-0.29±0.25	98±3	8
JZL 184	-0.14±0.11	82±4	8
L161982	-0.35±0.35	84±14	6
L-NAME	-0.07±0.03	97±10	6
Nimesulide	0.18±0.15	93.7±8	10
URB597	-0.20±0.19	76±10	7

Table 2.5 Effects of a range of inhibitors/antagonists on artery baseline tone and contractile responses, CBD studies
The effects of the inhibitors/antagonists used in CBD experiments on baseline tone and contractile responses. Δ alteration in baseline tone determined by the baseline tone after addition of inhibitor/antagonist minus baseline tone before addition of inhibitor/antagonist. Contractile tone to U46619 (10-300 nM) in combination with endothelin-1 (1-10 nM) is expressed as percentage of response to 124 mM KPSS. Values given ± s.e.m with *n* equal to the number of patients. Comparisons made using 1-way ANOVA with Dunnett's *post-hoc* test to compare all responses to time control.

Artery treatment	Baseline tone mN (Δ alteration)	Contractile Tone (% KPSS)	*n*
Time control	-0.24±0.09	68±4	37
AM251	-0.2±0.13	67±6	15
AM630	-0.31±0.12	60±7	8
Capsaicin	-0.9±0.31	56±4	7
Endothelium-denuded		70±7	14
Endothelium denuded + AM251		66±7	6
Indomethacin	-0.1±0.09	68±4	6
L-NAME	0.5±0.26	67±8	6
LY320135	-0.35±0.1	50±5	6
O-1918	-0.14±0.05	52±5	7

Table 2.6 Effects of a range of inhibitors/antagonists on artery baseline tone and contractile responses, AEA studies.
The effects of the inhibitors/antagonists used in AEA experiments on baseline tone and contractile responses. Δ alteration in baseline tone determined by the baseline tone after addition of inhibitor/antagonist minus baseline tone before addition of inhibitor/antagonist. Contractile tone to U46619 (10-300 nM) in combination with endothelin-1 (1-10 nM) is expressed as percentage of response to 124 mM KPSS. Values given ± s.e.m with *n* equal to the number of patients. Comparisons made using 1-way ANOVA with Dunnett's *post-hoc* test to compare all responses to time control. ** P<0.01

Artery treatment	Baseline tone mN (Δ alteration)	Contractile Tone (% KPSS)	*n*
Time control	-0.11±0.09	71±6	28
AM251	-0.36±0.2	70±11	7
AM630	0.18±0.13	67±9	5
Capsaicin	-0.9±0.4**	64±6	5
Endothelium-denuded		75±20	6
Indomethacin	0.03±0.1	72±4	7
L-NAME	-0.26±0.11	71±9	6
URB597	-0.16±0.24	82±14	5
O-1918	-0.08±0.11	53±6	6

2.8. Statistical Analysis

Unless otherwise stated, data are displayed as means with error bars representing s.e.m and *n* equalling the number of different patients. In contractile studies data points are derived from either the absolute response (mN) or the Δ value. In all relaxation studies, the percentage reduction in pre-imposed tone was used (see equation 1). In contractile and relaxant studies, sigmoidal concentration-response curves were fitted to these data using three-parameter logistic equation (bottom, top and EC_{50}) and a standard Hill slope of 1 in Prism (Version 5; GraphPad Software, La Jolla, California, USA). A standard Hill slope of 1 was chosen based on curve fit and given the low number of data points.

2.8.1. Statistical Analysis Used in Artery Storage

Second KPSS, U46619 and bradykinin responses were assessed for normal distribution using the Kolmogorov-Smirnov test. This revealed that these data were not normally distributed and therefore a Mann Whitney U test was used to compare arteries used fresh or those that had been stored overnight. To compare the potential effects of overnight storage on cannabinoid responses, data were tested for normal distribution using the Kolmogorov-Smirnov test. Data were normally distributed and therefore a two-way analysis of variance (2-way ANOVA) with a Bonferroni *post-hoc* test was used to compare whole data sets. $P<0.05$ was taken as significant.

2.8.2. Statistical Analysis Used in Cannabinoid Experiments

Vehicle control and cannabinoid test artery data sets were assessed for normal distribution using a Kolmogorov-Smirnov test. All data sets were found to have a normal distribution. Therefore, whole data sets were compared using 2-way ANOVA with Bonferroni *post-hoc* test. $P<0.05$ was taken as significant. In some experiments pEC_{50} and R_{max} were compared using 1-way ANOVA with Bonferroni *post-hoc* test.

2.8.3. Statistical Analysis Used in Analysis of Patient Notes

Cannabinoid concentration-response curves were compared between patients with various cardiovascular diseases/cardiovascular disease risk factors and those without. Artery data sets were assessed for normal distribution using a Kolmogorov-Smirnov test. All data sets

were found to have a normal distribution. Therefore, whole data sets were subsequently compared using 2-way ANOVA with Bonferroni *post-hoc* test. $P<0.05$ was taken as significant.

3. VASORELAXANT EFFECTS OF CANNABINOIDS IN HUMAN MESENTERIC ARTERIES

3.1. Introduction

The first evidence of the vasodilator effects of cannabinoids were shown using THC and AEA in rabbit cerebral arteries (Ellis *et al.*, 1995). This study found that both THC and AEA caused ≈25% vasodilation at concentrations between 0.1 and 1 mM (Ellis *et al.*, 1995). The effects of AEA but not THC were attributed to increases in the production of prostaglandin E_2 (PGE_2) and PGI_2 and could be inhibited using indomethacin (Ellis *et al.*, 1995). Since then much work has been carried out to support the vasorelaxant effects of cannabinoids in various vascular preparations, however efficacy, potency and mechanisms of action often differ depending on the cannabinoid, the species used or the vascular bed studied (reviewed in Randall *et al.*, 2004).

In vitro studies using rat third order mesenteric arteries showed that AEA causes maximal vasorelaxation that is partly endothelium-dependent and sensitive to CB_1 antagonism (White and Hiley 1997), with similar findings reported by O'Sullivan *et al* (2004). However, AEA efficacy is reduced in larger mesenteric arteries from the rat, which may be explained by the loss of an endothelial dependent component in these arteries (O'Sullivan *et al.*, 2004). The effects of AEA in larger mesenteric arteries or in the mesenteric bed, despite being endothelium-independent, are inhibited by rimonabant (Randall *et al.*, 1996; O'Sullivan *et al.*, 2004), desensitisation of the TRPV1 receptor (O'Sullivan *et al.*, 2004) or inhibition of potassium efflux (Randall *et al.*, 1996), implicating that the main vasorelaxant pathways in these preparations are activation of potassium channels and CB_1 and TRPV1 receptors. These studies found no role for the production of nitric oxide in AEA-induced vasorelaxation. In contrast, in small rat renal arteries, AEA caused vasorelaxation through CB_1- and nitric oxide-dependent pathways (Deutsch *et al.*, 1997). This study also showed that rat renal endothelial cells produce nitric oxide when stimulated with AEA via CB_1 activation.

The first work to study the effects of AEA in human arteries used the myometrial vascular bed and found that AEA did not cause vasorelaxation (Kenny *et al.*, 2002). In the human forearm

microcirculation, intra-arterial administration of AEA had no significant effect on blood flow, however dermal application of AEA (30 mM) increased forearm blood flow in a TRPV1 receptor-dependent manner (Movahed *et al.,* 2005). The only other study to look at the effects of AEA in human arteries is that of Kozlowska *et al.* (2007). This study showed that AEA caused maximal vasorelaxation of larger (2-4 mm outer diameter) human pulmonary arteries. The mechanisms of AEA action in the human pulmonary artery were not probed by Kozlowska *et al.* (2007).

2-AG causes maximal vasorelaxation of bovine coronary arteries, which is dependent on metabolism through MAGL, FAAH, COX and cytochrome P450 pathways (Gauthier *et al.,* 2005). In rat third order mesenteric arteries, 2-AG causes near maximal vasorelaxation, which can be enhanced to maximal vasorelaxation by incubating arteries with the COX-1 inhibitor flurbiprofen (Ho and Randall 2007). In rabbit mesenteric arteries, 2-AG caused near maximal vasorelaxation which is unaffected by inhibition of COX and nitric oxide, blockade of potassium channels or endothelial denudation, but is inhibited by high concentrations of CB_1 antagonist (Kagota *et al.,* 2001). By contrast, in rat isolated aortic rings 2-AG causes COX-mediated contractions (Stanke-Labesque *et al.,* 2004). There have been no reports of the effects of 2-AG in human arteries. However one study has reported that 2-AG causes release of nitric oxide in human artery endothelial cells (Stefano *et al.,* 2000).

THC causes half-maximal vasorelaxation of third order mesenteric arteries that is dependent on G protein-coupled receptors and ion channel modulation (O'Sullivan *et al.,* 2005). In the rat mesenteric vascular bed, the vasorelaxant effects of THC are reduced in arterial branches closer to the superior mesenteric artery, with THC causing contraction in the superior mesenteric artery (O'Sullivan *et al.,* 2005). In the rat aorta however, THC causes slight vasorelaxation with similar efficacy and potency to that of AEA (O'Sullivan *et al.,* 2005). In the rat aorta the effects of THC are inhibited by PTX, TRPV1 desensitisation and removal of the endothelium, whilst the effects of AEA are inhibited by PTX only (O'Sullivan *et al.,* 2005).

The potential vasorelaxation/vasoconstriction effects of CBD have not been fully reported in any vascular preparation. In arterial segments from the rat mesenteric vascular bed, CBD caused a

concentration-dependent near-maximal vasorelaxation (Offertaler *et al.*, 2003), but the underlying mechanisms were not probed. In several studies, including those involving human arteries, CBD has been proposed as an antagonist of a proposed novel endothelial cannabinoid receptor (Offertaler *et al.*, 2003; Kozlowska *et al.*, 2007).

Synthetic cannabinoid ligands have been reported to cause vasorelaxation. CP55,940 caused maximal vasorelaxation of rat third order mesenteric arteries, which was sensitive to CB_1 antagonism (White and Hiley 1998; O'Sullivan *et al.*, 2004). Vasorelaxation to CP55,940 was found to be endothelium-independent and had both receptor-mediated and direct action on ion channels (White and Hiley 1998). In rat aortae, CP55,940 caused maximal vasorelaxation sensitive to CB_2 antagonism (O'Sullivan *et al.*, 2005). Synthetic CB_2 specific ligands have also been reported to cause both hypotension and vasorelaxation. *In vivo*, HU-308 caused hypotension of rats that was inhibited by CB_2 receptor antagonism (Hanus *et al.*, 1999). JWH-015 caused maximal vasorelaxation of rat mesenteric arteries, which was mainly through inhibition of Ca^{2+} entry and was not inhibited by CB_2 antagonism (Ho and Hiley 2003). Topical application of JWH-015 or JWH-133 also caused slight or half-maximal vasorelaxation in rat knee synovial blood vessels (McDougall *et al.*, 2008). In this study CB_2 antagonism inhibited the effects of JWH-133 but not JWH-015 (McDougall *et al.*, 2008).

In summary, a wealth of research has shown that endocannabinoids, phytocannabinoids and synthetic cannabinoid ligands are capable of causing vasorelaxation in a range of vascular beds. However, few studies to date have investigated the effects of cannabinoids in human.

3.2. Aims

The aim of this study was to screen a range of cannabinoids in their ability to cause vasorelaxation of human mesenteric arteries. Screening experiments revealed 2-AG to be the most efficacious endocannabinoid tested. Therefore, a subsequent aim of this study was to characterise the underlying mechanisms of 2-AG-induced vasorelaxation.

3.3. Methods

3.3.1. Patient Consent and Arterial Preparation

In screening studies informed consent was taken from 25 patients for the use of mesenteric arteries, only 20 patients granted the use of their personal details and medical notes in this study. In further studies to characterise the effects of 2-AG, informed consent was taken from patients (n=44) for the use of mesenteric arteries, only 41 patients granted the use of their personal details and medical notes in this study. Patients were receiving colorectal re-sections for cancer (n=51) and inflammatory bowel disorders (n=10). As described in section 2.3 tissues were dissected, removing small mesenteric arteries that were either used fresh (n=36) or after overnight storage (n=33) as described in section 2.6

3.3.2. Myography Experiments

Arteries were mounted on a Mulvany-Halpern myograph subject to the normalisation and standard start procedures as described in sections 2.3 and 2.4. Cannabinoid concentration-response curves were carried out in viable arterial segments and compared to vehicle controls from an adjacent arterial segment from the same patient. To characterise the mechanisms of action of 2-AG, a range of pharmacological techniques were used as described in section 2.7. Comparisons were made between test and control arteries taken from the same patient.

3.3.3. *Post-hoc* Analysis

Post-hoc analysis was performed on control 2-AG responses from patients that had granted access to personal details and medical notes (n=41). 2-AG responses were analysed depending on the patient's medical history and medications. Particular attention was paid to diseases and medications that have either a direct vascular impact or are vascular disease risk factors.

3.3.4. Statistical Analysis

Mean percentage relaxation is displayed with error bars representing the s.e.m, and n equalling the number of patients. Sigmoidal concentration-responses curves with a standard Hill slope of 1 were fitted to those data using GraphPad Prism software. In vasorelaxation studies and analysis of patient disease state, BMI and medication comparisons were made between test and control vessel segments

from the same patient using 2-way ANOVA comparing the effects of treatment and log concentration. In $CaCl_2$ contraction studies comparisons were made using 1-way ANOVA. Significance was determined with $P<0.05$.

3.4. Results

3.4.1. Cannabinoid Screening

The characteristics of patients used in screening studies are given in Table 3.1. With the exception of the CB_2 agonist HU-308, all cannabinoids tested caused concentration-dependent vasorelaxation of human mesenteric arteries (see Table 3.2 and Figure 3.1). The maximal (R_{max}) response to CP55,940 was significantly greater than all other cannabinoids tested (Figure 3.2A). 2-AG was the most efficacious endocannabinoid tested and CBD was the most efficacious phytocannabinoid tested (Figure 3.2B). All cannabinoids caused vasorelaxation with equal potency with the exception of CBD, which had statistically lower potency than both THC and AEA (Figure 3.2A).

Table 3.1 Patient characteristics screening studies

Characteristic	Range	Mean ± s.e.m
Ethnicity	20 UK white	
Male	18	
Female	2	
Age	48-85	72±2
Weight (kg)	50-113	84 ± 4
BMI (kg/m^2)	20-39	28±1
Artery size (μm)	375-1257	698±65
Bradykinin response (% reduction in pre-imposed tone)	72-110	85±2
Smoking habits		
Non smokers	17	
0 - 10 CPD	2	
10 - 20 CPD	1	
> 20 CPD	0	
Drinking habits		
< 10 units p/w	10	
10 - 20 units p/w	6	
> 20 units p/w	4	
Operation		
Right hemicolectomy	8	
Left hemicolectomy	4	
Sigmoid colectomy	1	
Anterior resection	4	
Abdominoperineal resection	2	
Total colectomy	1	
Reason for surgery		
Cancer	19	
Inflammatory bowel disorder	1	
Dukes staging		
Dukes A	8	
Dukes B	6	
Dukes C	5	
Dukes D	0	
Systolic blood pressure (mm/Hg)	117-172	147±3
Diastolic blood pressure (mm/Hg)	65±101	81±2
Type-2 diabetic	10	
Heart disease	10	
Heart failure	0	
Hypercholesterolaemia	13	
Hypertensive	13	
α-1 adrenoceptor antagonist (total)	1	
Alfuzosin	0	
Terazosin	1	
ACE inhibitors (total)	6	
Lisinopril	2	
Ramipril	4	
AT1 receptor antagonists (total)	2	
Losartan	1	
Valsartan	1	

Characteristic	Range	Mean ± s.e.m
Beta blockers (total)	5	
Metoprolol	2	
Atenolol	2	
Bisoprolol	1	
Calcium channel blocker (total)	6	
Amlodipine	3	
Nifedipine	2	
Lodipine	1	
Digoxin	1	
Diuretics (total)	2	
Furosemide	2	
GTN	5	
Hypoglycaemic medication (total)	12	
Gliclazide	5	
Metformin	7	
NSAID medication (total)	14	
Aspirin	9	
Ibuprofen	1	
Paracetamol	4	
Co Codamol	0	
Statin (total)	11	
Atorvastatin	2	
Simvastatin	9	
Pravastatin	0	
Thiazolidinedione (total)	2	
Pioglitazone	2	

Table 3.2 Effects of cannabinoids in human mesenteric arteries. Comparisons made between concentration response curve and vehicle control curve using 2-way ANOVA. *** P<0.001.

Cannabinoid	R_{MAX}	pEC_{50}	*n*	sig
AEA	28±2.3	5.9 ± 0.2	12	***
2-AG	41.8±2.6	5.5±0.1	12	***
THC	26.6±2.4	6±0.2	12	***
CBD	38.6±3.6	5.2±0.2	12	***
CP55,940	56.6±3.1	5.4±0.1	12	***
HU308	Did not cause vasorelaxation		5	

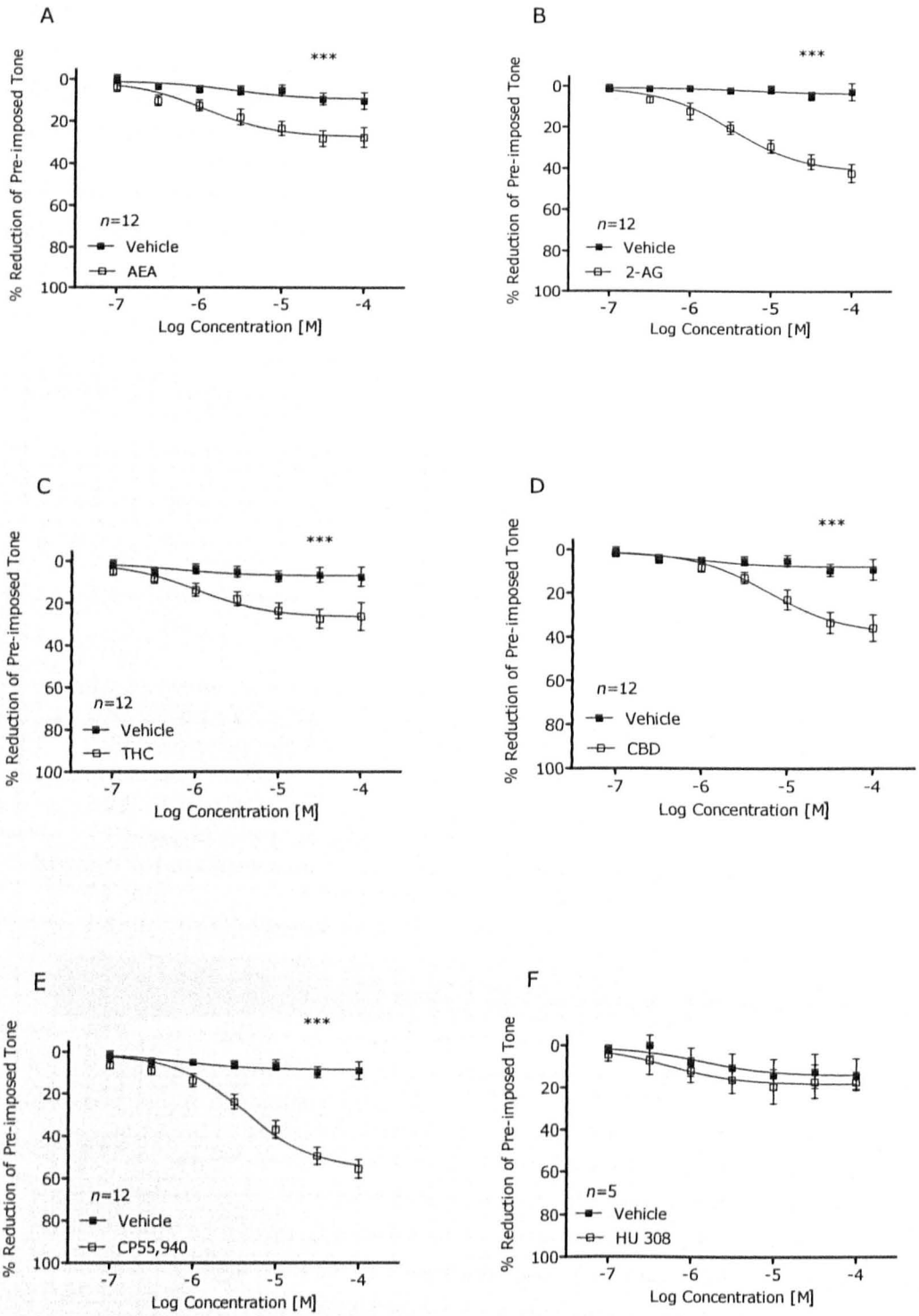

Figure 3.1. Screening concentration-response curves
Concentration-response curves showing the effects of cannabinoids in human mesenteric arteries. Points represent means with error bars representing the s.e.m. Comparisons between curves were made using 2-way ANOVA. *** P<0.001.

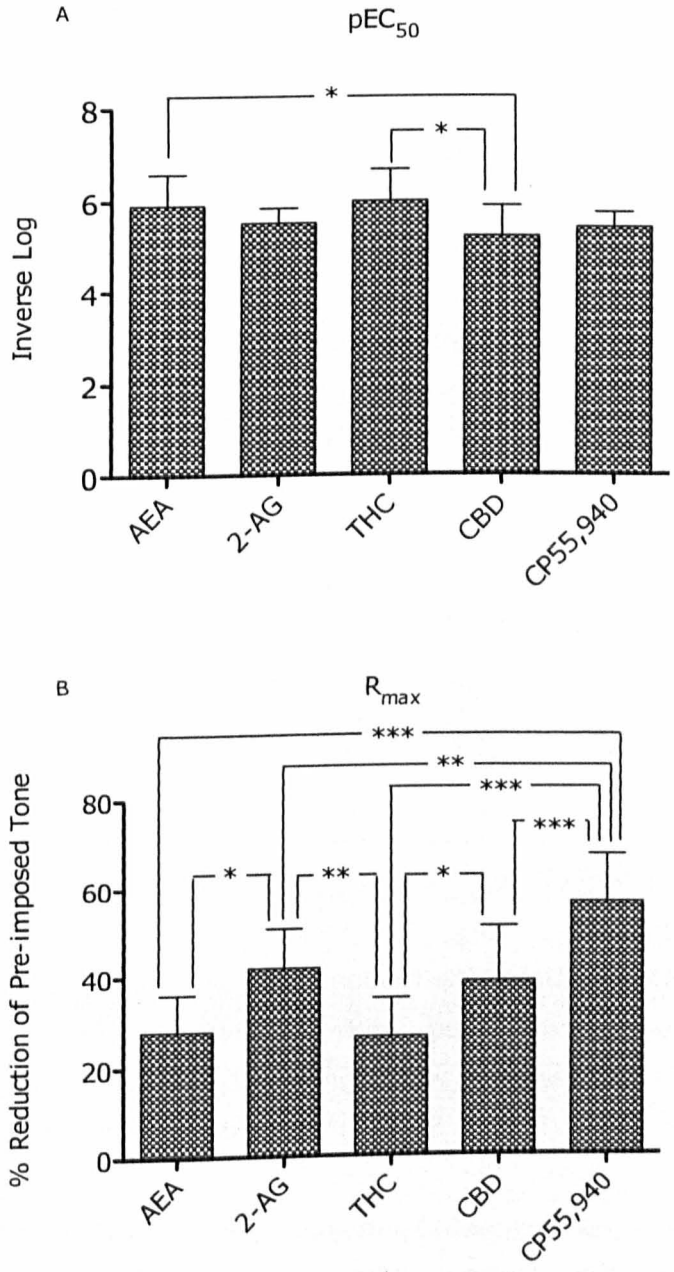

Figure 3.2 Cannabinoid efficacy and potency
Comparisons of cannabinoid pEC_{50} (A) and R_{max} (B) values in human mesenteric arteries. Comparisons made using 1-way ANOVA with Bonferroni *post-hoc* test. $P<0.05$ *, $P<0.01$ ** and $P<0.001$ ***.

3.4.2. Characterisation of the Vasorelaxant Effects of 2-Arachidonoylglycerol

44 patients (table 3.3) were used in further 2-AG vasorelaxation studies, in these patients 2-AG caused vasorelaxation with an R_{max} of 67 ± 3% and a pEC_{50} of 5.5 ± 0.1 (Figure 3.3A). 2-AG caused a modest reduction in baseline tone (Figure 3.3B).

Antagonism of the CB_1 (AM251, 100 nM) or CB_2 (AM630, 100 nM) receptors (Figure 3.4A and B), desensitisation of the TRPV1 receptor using capsaicin (10 μM) (Figure 3.4C) had no effect on 2-AG-induced vasorelaxation. Removal of the endothelium or incubation of endothelium-intact arterial segments with L-NAME (300 μM) had no effect on vasorelaxation to 2-AG (Figure 3.5A and B). Incubation with the MAGL inhibitor JZL184 (1 μM) or the FAAH inhibitor URB597 (1 μM) had no effect on 2-AG-induced vasorelaxation (Figure 3.5C and D).

Incubation with the non-selective COX inhibitor indomethacin (10 μM) significantly reduced vasorelaxation to 2-AG (Figure 3.6A). The COX-1 favourable inhibitor flurbiprofen (10 μM) but not the COX-2 inhibitor nimesulide (10 μM) inhibited 2-AG-induced vasorelaxation (Figure 3.6B & C). Antagonism of the prostanoid IP (Cay10441, 100 nM) and EP_4 (L-161,982, 1 μM) receptors significantly reduced the vasorelaxant responses to 2-AG (Figure 3.6D & E). However, the prostanoid EP_1, EP_2, EP_3, DP and TP receptor antagonist (AH6809, 1 μM) potentiated the response to 2-AG (Figure 3.6F).

In arteries contracted with U46619 in Ca^{2+}-free PSS, 2-AG-induced vasorelaxation was significantly inhibited compared to PSS containing Ca^{2+} (Figure 3.7A). In arteries contracted with KPSS solution to inhibit potassium efflux and allow voltage gated calcium influx, 2-AG-induced vasorelaxation was inhibited (Figure 3.7B). In arteries bathed in high potassium Ca^{2+}-free solution and incubated with 2-AG, the maximum contraction to $CaCl_2$ was inhibited in arterial segments incubated with 100 μM, but not 10 μM 2-AG (Figure 3.7C).

Table 3.3 Patient characteristics, diagnosis and medications 2-AG study

Characteristic	Range	Mean ± s.e.m
Ethnicity	44 UK white	
Male	30	
Female	11	
Age	27 - 82	68 ± 2
Weight (kg)	50 - 117	82 ± 3
BMI (kg/m^2)	20 - 39	28 ± 1
Vessel size (μm)	302 - 1309	668 ± 40
Bradykinin response (% reduction in pre-imposed tone)	71 - 110	89 ± 2
Smoking habits		
Non smokers	30	
0 - 10 CPD	2	
10 - 20 CPD	8	
> 20 CPD	1	
Drinking habits		
< 10 units p/w	21	
10 - 20 units p/w	5	
> 20 units p/w	5	
Operation		
Right hemicolectomy	14	
Left hemicolectomy	7	
Sigmoid colectomy	2	
Anterior resection	11	
Abdominoperineal resection	1	
Total colectomy	6	
Reason for surgery		
Cancer	32	
Inflammatory bowel disorder	9	
Dukes staging		
Dukes A	11	
Dukes B	11	
Dukes C	10	
Dukes D	0	
Systolic blood pressure (mm/Hg)	110 - 188	150 ± 3
Diastolic blood pressure (mm/Hg)	65 ± 110	86 ± 1
Type-2 diabetic	10	
Heart disease	9	
Heart failure	0	
Hypercholesterolaemia	16	
Hypertensive	16	
α-1 adrenoceptor antagonist (total)	1	
Alfuzosin	1	
Terazosin	0	

Characteristic	Range	Mean ± s.e.m
ACE inhibitors (total)	6	
Lisinopril	4	
Ramipril	2	
AT1 receptor antagonists (total)	2	
Losartan	1	
Irbesartan	1	
Beta blockers (total)	8	
Metoprolol	2	
Atenolol	5	
Bisoprolol	1	
Calcium channel blocker (total)	4	
Amlodipine	2	
Nifedipine	0	
Lodipine	2	
Digoxin	1	
Diuretics (total)	2	
Furosemide	2	
GTN	5	
Hypoglycaemic medication (total)	6	
Gliclazide	2	
Metformin	4	
Nsaid medication (total)	14	
Aspirin	10	
Ibuprofen	1	
Paracetamol	2	
Co Codamol	1	
Statin (total)	16	
Atorvastatin	4	
Simvastatin	12	
Pravastatin	0	
Thiazolidinedione (total)	1	
Pioglitazone	1	

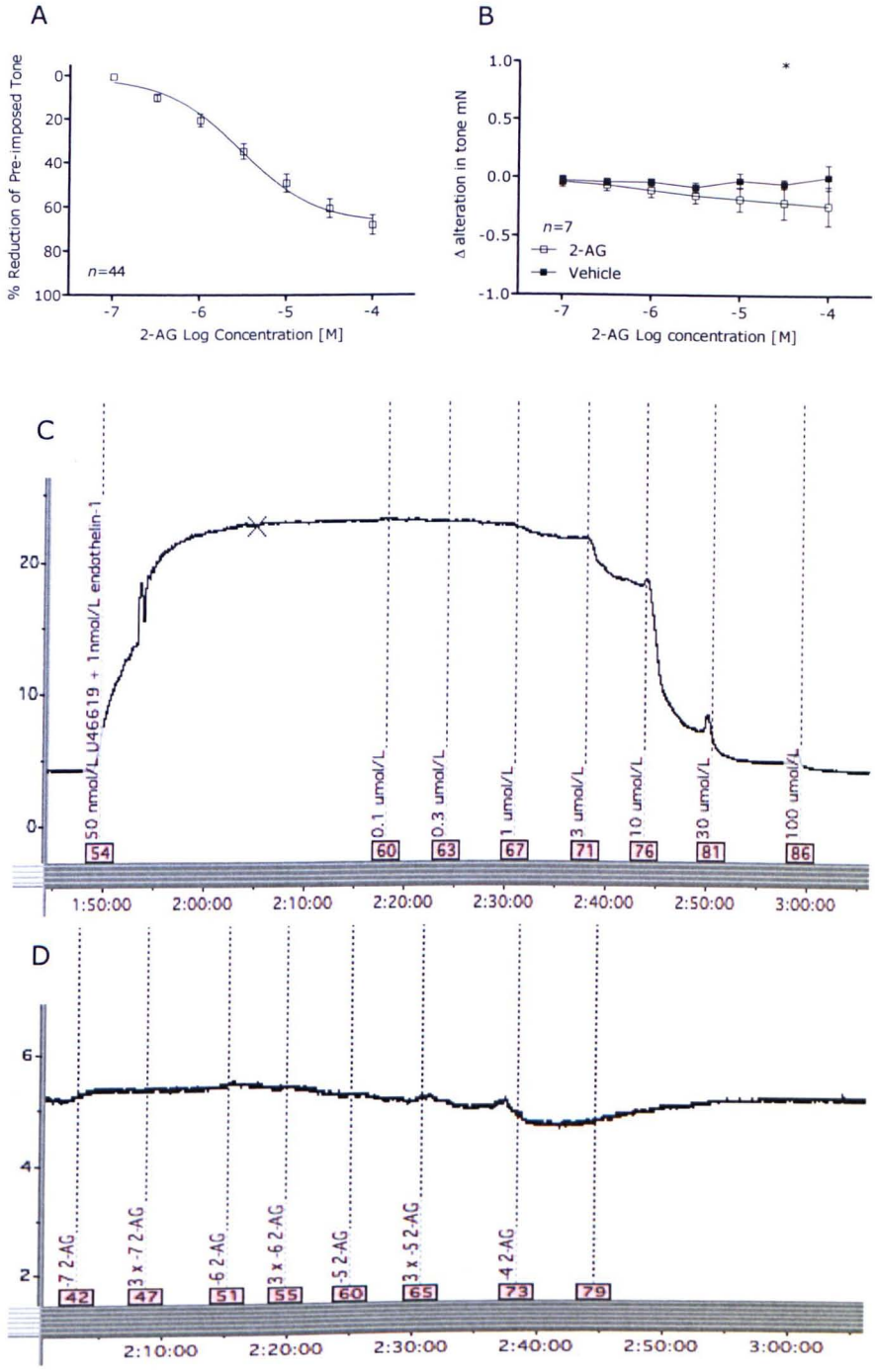

Figure 3.3 2-AG responses
2-AG-induced vasorelaxation in all patient samples with representative trace (A and C). Effects of 2-AG on baseline compared to vehicle control and representative trace (B and D). Comparisons made using 2-way ANOVA. * $P<0.05$.

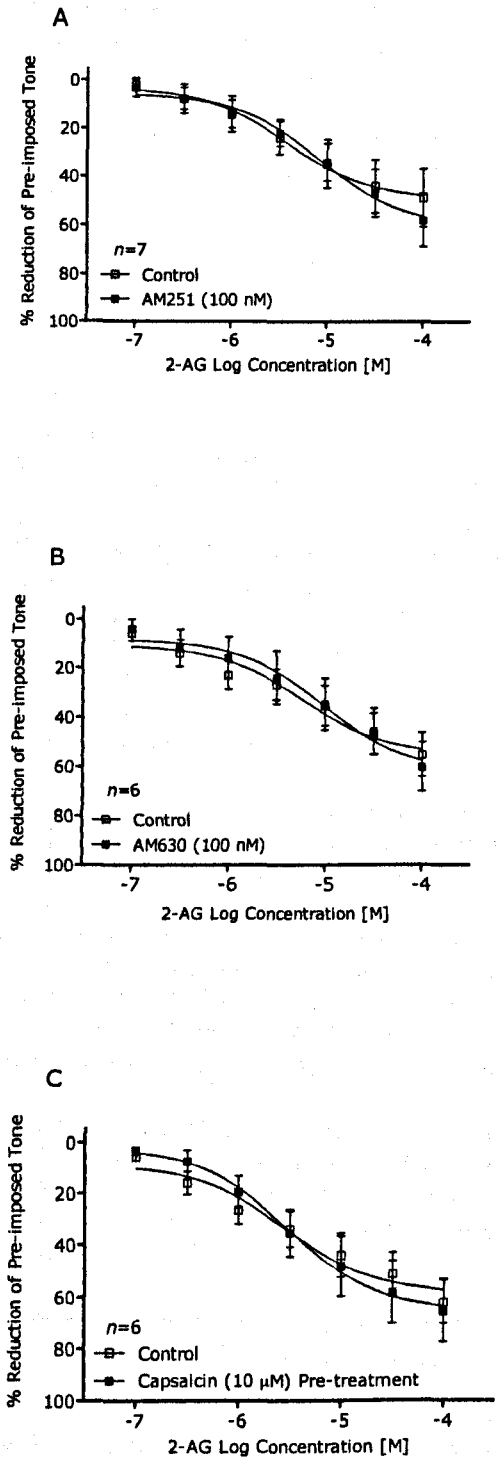

Figure 3.4 Potential receptor involvement in 2-AG responses
2-AG-induced vasorelaxation in arteries incubated with the CB_1 antagonist AM251 (A), in arteries incubated with the CB_2 antagonist AM630 (B) or in arteries where the TRPV1 receptor had been desensitised after 1-hour incubation with the TRPV1 agonist capsaicin (C). Comparisons made using 2-way ANOVA

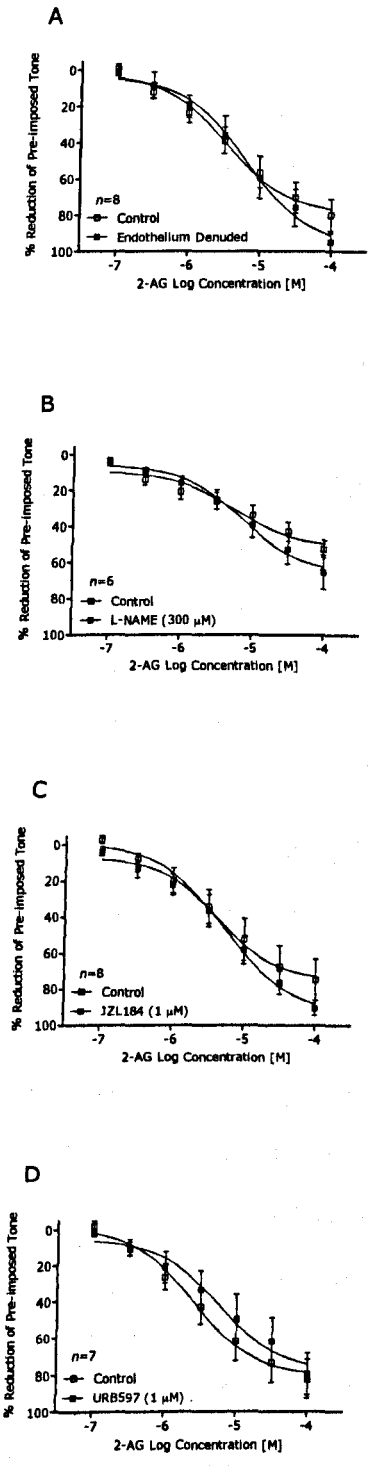

Figure 3.5 Potential involvement of the endothelium in 2-AG responses 2-AG induced vasorelaxation in arteries after endothelium denudation (A), in arteries incubated with the nitric oxide synthase inhibitor L-NAME (B), in arteries incubated with the MAGL inhibitor JZL184 (C) or in arteries incubated with the FAAH inhibitor URB597 (D). Comparisons made using 2-way ANOVA.

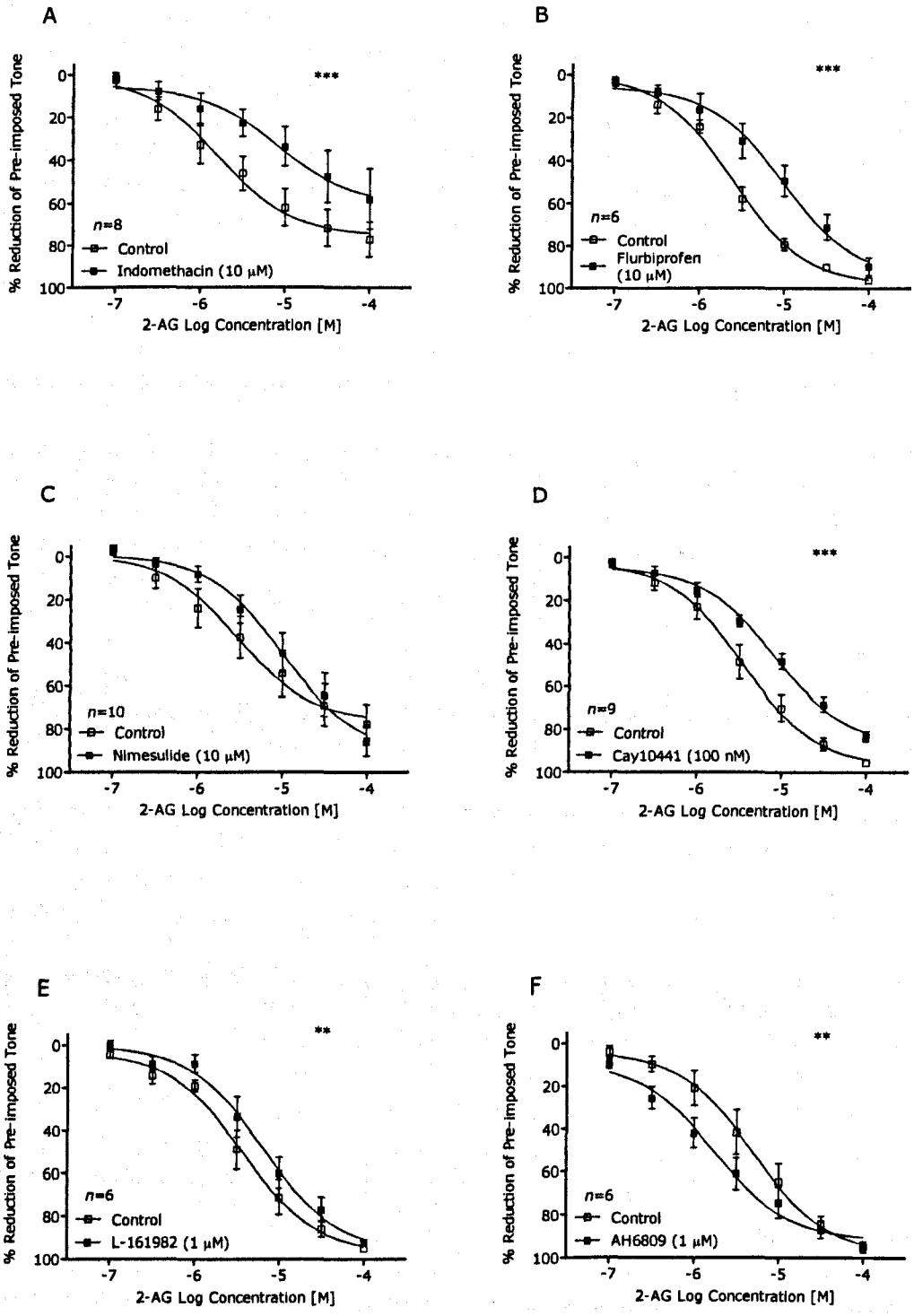

Figure 3.6 Potential COX involvement in 2-AG responses
2-AG induced vasorelaxation in arteries incubated with the non-selective COX inhibitor indomethacin (A), the COX-1 favourable inhibitor flurbiprofen (B) and the COX-2 inhibitor nimesulide (C). 2-AG-induced vasorelaxation in the presence of the prostanoid IP receptor antagonist Cay10441 (D), the prostanoid EP_4 receptor antagonist L-161982 (E) and the prostanoid EP_1, EP_2, EP_3, DP and TP antagonist AH6809. Comparisons made using 2-way ANOVA. ** $P<0.01$ and *** $P<0.001$.

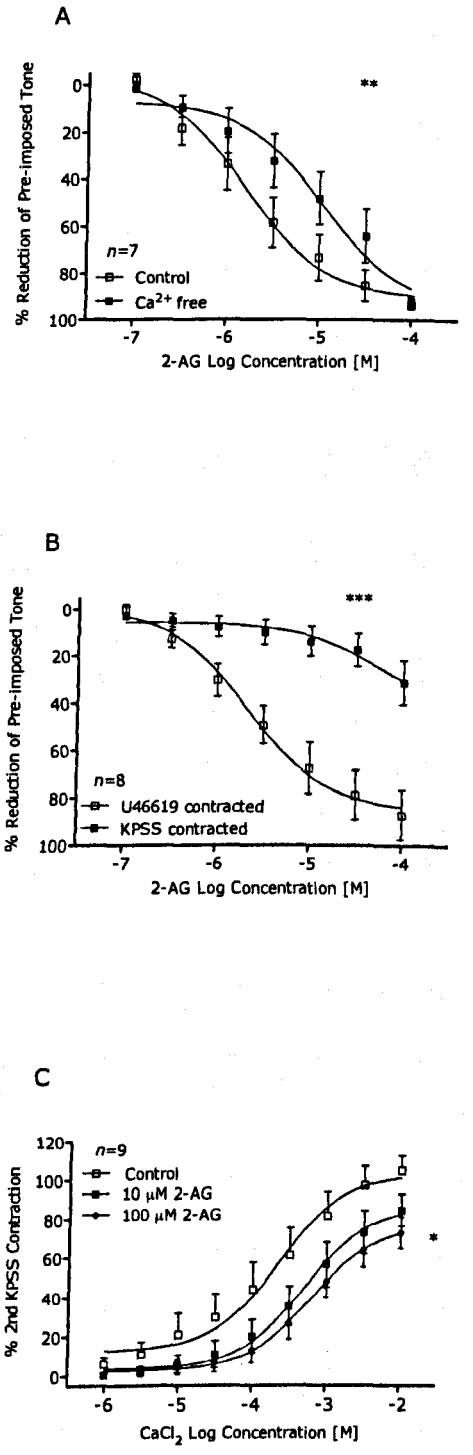

Figure 3.7 Potential ion channel involvement in 2-AG responses
2-AG-induced vasorelaxation in arteries incubated with $Ca2^{+}$-free PSS (A) or in arteries contracted with KPSS (B). $CaCl_2$ contractions in arterial segments incubated with 2-AG (C). Comparisons of vasorelaxation made using 2-way ANOVA. Comparisons of $CaCl_2$ made comparing R_{max} values using 1-way ANOVA, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

3.4.3. *Post-hoc* Analysis of Patient Medical Records

Due to the variability of control responses to 2-AG among patients (response range at 100 μM 2-AG, 0–103% relaxation), *post-hoc* analysis of patient responses were compared between a variety of variables such as diagnosis, current medication and cardiovascular risk factors.

2-AG responses were not correlated with age but were reduced in males compared to females (Figure 3.8A & D). There was no correlation between the maximal responses to 2-AG and the patient's vasorelaxant response to bradykinin (Figure 3.8B). 2-AG responses were not different in patients with cancer and those without (Figure 3.8C). 2-AG responses were significantly reduced in patients diagnosed with heart disease (previous myocardial infarction and/or ischaemic heart disease), hypercholesterolaemia and type-2 diabetes (Figure 3.9A, B & C), but not those with hypertension, a BMI >25 kg/m^2 or smokers (Figure 6D, E, & F). 2-AG responses were reduced in those taking NSAIDs, statins and hypoglycaemic medication (Figure 3.10A, B & C) but not those taking beta-blockers or ACE inhibitors (Figure 3.10D & E).

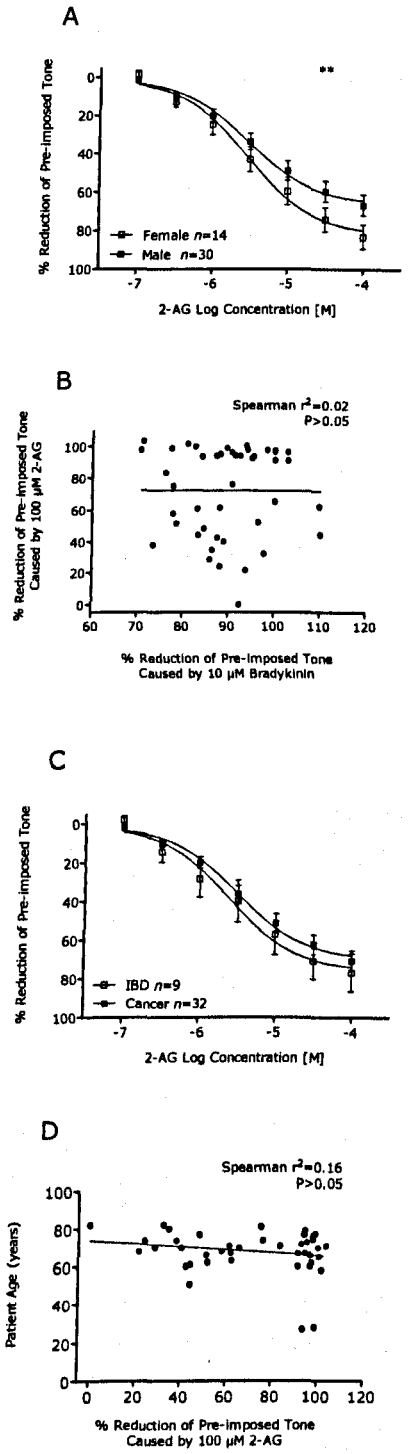

Figure 3.8 Patient characteristics and 2-AG responses.
Responses to 100 µM 2-AG correlated with patient age (A) and 2-AG-induced vasorelaxation compared between male and female patients (B). 100 µM 2-AG responses correlated with 10 µM bradykinin response (C). 2-AG responses in patients with cancer vs. those without (D). Comparisons made using 2-way ANOVA and correlations made using Spearman correlation coefficient, ** $P<0.01$.

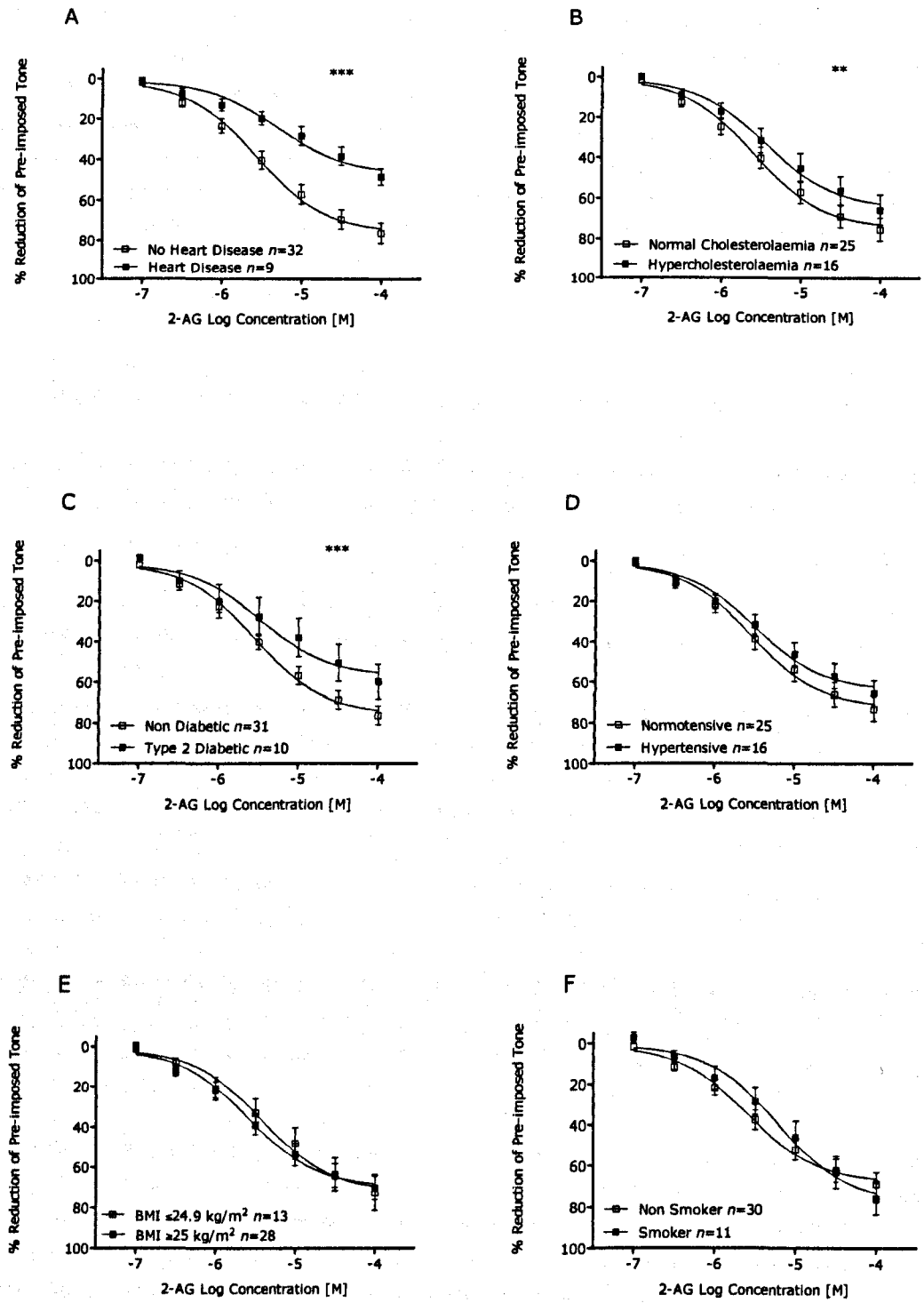

Figure 3.9 Cardiovascular disease/disease risk factors and 2-AG responses

2-AG concentration-response curves in patients with heart disease (A), hypercholesterolaemia (B), type-2 diabetes (C), hypertension (D), elevated BMI (E) and in patients who smoke (F). Comparisons were made between patients with a given characteristic and those without using 2-way ANOVA. ** $P<0.01$ and *** $P<0.001$.

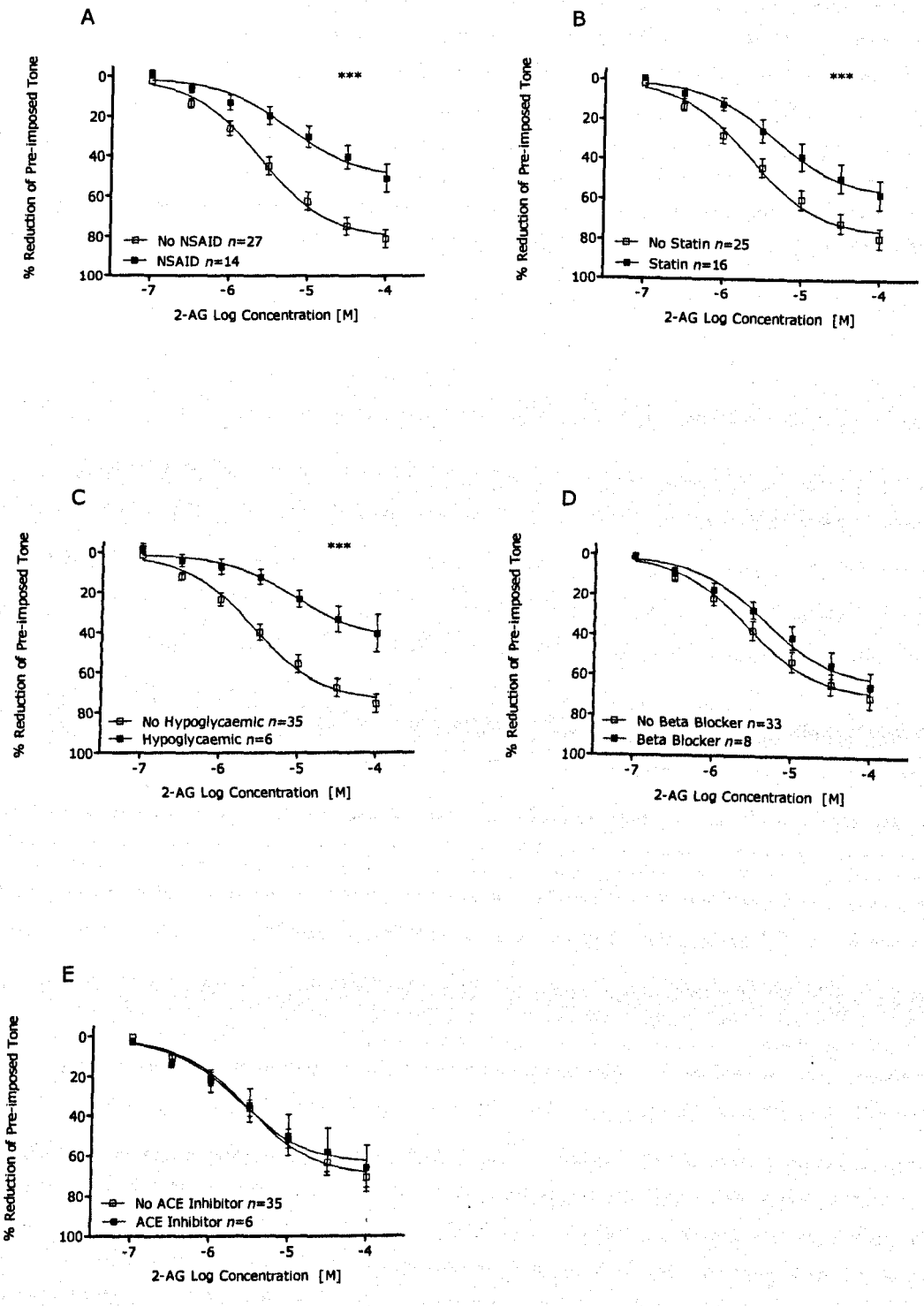

Figure 3.10 Patient medication and 2-AG responses
2-AG concentration-response curves in patients taking NSAIDs (A), statins (B), hypoglycaemic (C), beta-blocking (D) and ACE inhibiting medication (E). Comparisons were made between patients taking a given medication and those that were not using 2-way ANOVA. *** $P<0.001$.

3.5. Discussion

The aim of this study was to screen the effects of a range of cannabinoids in human mesenteric arteries. This study reports that all cannabinoids tested, with the exception of the CB_2 agonist, cause vasorelaxation of human mesenteric arteries. Given that 2-AG was the most efficacious endocannabinoid tested, the mechanisms of action this ligand were probed. These experiments revealed that 2-AG-induced vasorelaxation was dependent on COX metabolism, prostanoid receptor activation, extracellular calcium and ion channel modulation. *Post-hoc* analysis of patient notes revealed that 2-AG responses were blunted in a range of cardiovascular diseases or cardiovascular disease risk factors.

This study has shown that AEA, 2-AG, THC, CBD and CP55,940 but not HU308 cause concentration-dependent vasorelaxation of human mesenteric arteries. The initial screening experiments revealed vasorelaxation to all cannabinoids was reduced in efficacy compared to mesenteric arteries from animal models. In rat mesenteric arteries, AEA caused maximal vasorelaxation with a pEC_{50} in the low micromolar range (White and Hiley 1997; O'Sullivan *et al.,* 2004), yet in human mesenteric arteries AEA causes only a small degree of vasorelaxation with similar low micromolar potency. In rat mesenteric arteries, 2-AG is reported to have a pEC_{50} in the low micromolar range and cause near maximal vasorelaxation (Ho and Randall 2007), yet in human mesenteric arteries 2-AG causes a half maximal vasorelaxation with similar low micromolar potency. In rat mesenteric arteries, THC is reported to have a pEC_{50} in the low micromolar range and cause half-maximal vasorelaxation (O'Sullivan *et al.,* 2005), yet in human mesenteric arteries THC causes only slight vasorelaxation with similar low micromolar potency. In the rat mesenteric arteries, CP55,940 is reported to have a pEC_{50} in the low micromolar range and cause maximal vasorelaxation (White and Hiley 1998; O'Sullivan *et al.,* 2004), yet in human mesenteric arteries CP55,940 causes half-maximal vasorelaxation with similar low micromolar potency. In rat mesenteric arteries CBD causes maximal vasorelaxation, however potency was not reported (Offertaler *et al.,* 2003). Given, that CBD potency was not reported in the previous study, comparisons couldn't be made, however comparisons of efficacy show that vasorelaxation seen in human mesenteric arteries is less than seen by Offertaler *et al.*, (2003).

Comparisons between efficacy and potency of HU308 cannot be made, as these values have not previously been determined. Reduced cannabinoid potency in human pulmonary artery has previously been reported (Kozlowska *et al.*, 2007; Kozlowska *et al.*, 2008). Inter-species variability in cannabinoid potency and efficacy has previously been reported, for example in rat coronary arteries AEA causes approximately half maximal vasorelaxation (White *et al.*, 2001) whereas AEA does not have a vasorelaxant effect on porcine coronary arteries (Fleming *et al.*, 1999). Therefore, reduced responses might be ascribed to the differences in species. However, the patients who took part in this study had a high average age. Ageing is associated with decreased vascular smooth muscle and endothelial function (reviewed by Byung Pal and Hae Young 2001; Taddei *et al.*, 2006), and has been used to explain the reduced efficacy seen between acetylcholine relaxation in humans and rats (Hutri-Kahonen *et al.*, 1999). The mean age of participants in this study was 72 and the mean age of participants in both works of Kozlowska *et al.* (2007; 2008) were approximately 60 years old. Therefore, the reduced efficacy observed could be attributed to the participant's age. However, 2-AG responses were not negatively correlated with patient age, which suggests 2-AG efficacy is not dependent on age.

In animal arteries, AEA causes vasorelaxation of near maximal magnitudes and often displays greater efficacy than 2-AG. However, in the present study 2-AG caused statistically greater vasorelaxation than that of AEA. The disparity between the findings of this study and those in the rat mesenteric vasculature (O'Sullivan *et al.*, 2004) or human pulmonary artery (Kozlowska *et al.*, 2007) may be attributed to species differences. However, in this study arteries were taken from a patient population that presented with at least one medical condition, therefore this factor could have masked/reduced AEA-induced vasorelaxation. For example, Ho *et al.* (2007) observed that AEA-induced vasorelaxation could be potentiated if COX-2 and FAAH enzymes were inhibited, which may be true in humans, especially in conditions where COX and FAAH may be altered.

The R_{max} of CP55,940 was significantly greater than all other cannabinoids tested, whilst HU308 was ineffective as a vasorelaxant. Given that CP55,940 is highly potent at both CB_1 and CB_2 receptors (Showalter *et al.*, 1996) and HU308 is a potent CB_2 agonist that is

devoid of CB_1 activity (Hanus *et al.,* 1999), this could suggest that the CB_1 receptor, but not the CB_2 receptor may contribute to vascular tone in the human mesenteric arteries.

Given that initial screening experiments revealed 2-AG to be the more efficacious endocannabinoid tested, further work was carried out to establish the underlying mechanisms of action. In a larger patient sample 2-AG induced vasorelaxation of human mesenteric arteries with R_{max} and pEC_{50} values of 67 ± 3% and 5.5 ± 0.1, n=44. This is a similar potency and efficacy to that observed in rat mesenteric arteries (Ho and Randall 2007), but lower than that observed in rabbit mesenteric arteries (Kagota *et al.,* 2001). It should be noted that the vasorelaxation reported in this study is above reported physiological and pathophysiological levels of 2-AG (Quercioli *et al.,* 2011). However, 2-AG is an unstable compound (Rouzer *et al.,* 2002) that is produced on demand to function in a paracrine fashion (Maccarrone 2008), therefore, it is likely that plasma concentrations of 2-AG may not actually reflect cellular 2-AG concentrations.

This study has shown that 2-AG-induced vasorelaxation was not dependent on activation of CB_1, CB_2 or TRPV1 receptors which has been shown to partially underpin the vasoactive effects of other cannabinoid ligands in the mesenteric arteries of rats (O'Sullivan *et al.,* 2005). 2-AG activation of the CB_1 receptor causes vasorelaxation of rat cerebral arteries (Hillard *et al.,* 2007) and rabbit mesenteric arteries (Kagota *et al.,* 2001; Hillard *et al.,* 2007). Also, despite the findings that, under some conditions, 2-AG binding affinity for the CB_2 receptor is enhanced (Ben-Shabat *et al.,* 1998), and that 2-AG has been shown to stimulate Ca^{2+} influx through the TRPV1 receptors in human brain endothelial cells (Golech *et al.,* 2004), the potential role of these two receptors in 2-AG-induced vasorelaxation was previously untested. In the present study, 2-AG-induced vasorelaxation of human mesenteric arteries was not found to be mediated by these receptors, although all are present in human vascular smooth muscle cells (Sugiura *et al.,* 1998; Rajesh *et al.,* 2008; Wang *et al.,* 2008) and endothelial cells (Fantozzi *et al.,* 2003; Rajesh *et al.,* 2007).

In rat mesenteric arteries, vasorelaxation to 2-AG has been shown to be endothelium-dependent (Ho and Randall 2007). However, this study reports that 2-AG-induced vasorelaxation is not inhibited by removal of the endothelium or inhibition of nitric oxide. Furthermore,

the maximal vasorelaxant response to 2-AG was not correlated with the endothelium-dependent response to bradykinin in the same patient. A lack of a role for the endothelium or nitric oxide production in the vascular response to 2-AG in human mesenteric arteries is in agreement with previous findings in rabbit mesenteric arteries (Kagota *et al.,* 2001), but not in rat mesenteric arteries (Ho and Randall 2007) or bovine coronary arteries (Gauthier *et al.,* 2005), possibly highlighting species differences in 2-AG vascular responses.

2-AG is reported to be an unstable compound (Rouzer *et al.,* 2002), which can be hydrolysed by MAGL and FAAH (Muccioli 2010). Hydrolysis of 2-AG, potentially through both these pathways has previously been shown to cause vasorelaxation of bovine coronary arteries (Gauthier *et al.,* 2005). However, this study reports that 2-AG-induced vasorelaxation is not mediated by 2-AG hydrolysis through MAGL and FAAH. COX-1 and -2 are expressed in human endothelium and smooth muscle cells (Zidar *et al.,* 2009), and 2-AG serves as a substrate for COX metabolism (Kozak *et al.,* 2000; Rouzer and Marnett 2005). Furthermore, COX metabolism has been implicated in vasorelaxation to 2-AG in the bovine coronary artery (Gauthier *et al.,* 2005), but not rat or rabbit mesenteric arteries (Kagota *et al.,* 2001; Ho and Randall 2007). This study examined any potential role for COX metabolism and found that 2-AG-induced vasorelaxation of the human mesenteric artery is inhibited by the non-selective COX inhibitor indomethacin. To further explore the specific COX isoform, the COX inhibitor flurbiprofen, which displays greater affinity for COX-1, and the selective COX-2 inhibitor nimesulide, were used (Warner *et al.,* 1999). Flurbiprofen, but not nimesulide, caused inhibition of 2-AG-induced vasorelaxation, suggesting that 2-AG-induced vasorelaxation preferentially occurs through COX-1-dependent pathways in this model. In contrast, in the rat mesenteric artery 2-AG-induced vasorelaxation is limited by COX-1 metabolism (Ho and Randall 2007). Therefore, 2-AG-induced vasorelaxation that is COX-1 dependent is a novel finding that may be unique to humans. This finding also shows disparity between 2-AG pathways in rat and human mesenteric arteries and therefore suggests the need for further human work.

COX metabolism of 2-AG produces a range of novel prostanoid glycerol esters (Nirodi *et al.,* 2004). Prostanoid glycerol esters, in human and rat plasma, are reported to undergo hydrolysis to

prostanoids (Kozak *et al.,* 2001). However, the rate of hydrolysis depends on the assay conditions. For example in rat and human plasma the half-life of prostanoid glycerol esters is reported to be 16 seconds and 16 minutes respectively (Kozak *et al.,* 2001). However, in cerebral spinal fluid and in the presence of RAW264.7 macrophages prostaglandin glycerol esters are reported to be stable (Kozak *et al.,* 2001; Nirodi *et al.,* 2004). Therefore, given the potential for prostanoid production, further experiments investigated potential roles for the DP_1, EP_2, EP_4 and IP prostanoid receptors, which are all reported to cause vasorelaxation (Coleman *et al.,* 1994; Norel 2007). This study found that 2-AG-induced vasorelaxation is dependent on activation of the prostanoid EP_4 and IP receptors. In the rat aorta, the endocannabinoid AEA has been shown to cause vasorelaxation through multiple pathways including activation of prostanoid EP_4 receptors (Herradón *et al.,* 2007). Since prostaglandin glycerol esters are unable to activate prostanoid receptors (Nirodi *et al.,* 2004), it is likely that COX metabolism of 2-AG results in PGE_2 and PGI_2 formation via prostaglandin glycerol ester production and subsequent hydrolysis to prostanoids. The finding that AH6809 potentiated 2-AG induced vasorelaxation could be explained due to AH6809 having low micromolar affinity for contractile prostanoid receptors (Abramovitz *et al.,* 2000). Metabolism of 2-AG through COX-1 may result in formation of both contractile and relaxant prostanoids. It is therefore likely that AH6809 has antagonised any potential activity of contractile prostanoids.

Cannabinoids cause vasorelaxation partly through ion channel modulation (Randall *et al.,* 1997), which was investigated in the present study. When arteries were contracted using high potassium PSS, 2-AG-induced vasorelaxation was inhibited, suggesting that 2-AG-induced vasorelaxation is not primarily through inhibition of VOCCs and may be dependent on K^+ channel activation. This is a finding common with a number of cannabinoid ligands in a range of vascular preparations (Randall *et al.,* 1997; White *et al.,* 2001), including human arteries (Kozlowska *et al.,* 2007). To determine a potential role for calcium channels, some arterial segments were contracted using U46619 in calcium-free PSS. Under these conditions, partial inhibition of 2-AG-induced vasorelaxation was observed, suggesting that 2-AG-induced vasorelaxation is in part

calcium-dependent. Cannabinoids also cause vasorelaxation via inhibition of Ca^{2+} entry through voltage-operated calcium channels (White and Hiley 1998). In the present study, 2-AG, at the highest concentrations, inhibited the maximum contraction to calcium chloride, suggesting that 2-AG can inhibit calcium entry, and may explain the slight vasorelaxation observed in KPSS contracted arteries. Taken together, these findings show that 2-AG modulates some ion channels to favour vasorelaxation, which may be either coupled to prostanoid receptor activation or a direct action.

Given that the patient population in this study is elderly and has a range of medical problems, *post-hoc* analysis of the control 2-AG responses in each patient were carried out to establish if these variables influenced the effects of 2-AG. No correlation between 2-AG responses and patient age was observed, however 2-AG responses were blunted in males compared to females. In rat mesenteric arteries non-endothelium-dependent vasorelaxation is reduced in males compared to females (Keung *et al.*, 2005). This reduction is attributed to over activation of contractile prostaglandin receptors and is reversed by 17-beta estradiol (Keung *et al.*, 2005).

In patients with chronic heart failure, plasma 2-AG levels are reported in the mid-nanomolar range when compared to low nanomolar concentrations in healthy controls (Weis *et al.*, 2010), similarly 2-AG levels are measured at high nanomolar concentrations in patients with acute myocardial infarction compared to mid-nanomolar concentrations in healthy patients (Wang *et al.*, 2011). Local tissue levels of 2-AG are increased in the aorta and visceral adipose tissue in atherosclerotic mice (Montecucco *et al.*, 2009). To try and understand the potential significance of altered levels of 2-AG on the vasorelaxant response to 2-AG, *post-hoc* analysis was performed in patients with or without cardiovascular disease or risk factors. This study found that patients diagnosed with heart disease, diabetes and hyperlipidaemia had blunted responses to 2-AG when compared to patients without the diseases. It is known in all these disease states that prostaglandin pathways are often dysfunctional and often associated with increased production of COX-derived contractile prostaglandins (Goodwill *et al.*, 2008; Goodwill *et al.*, 2008), increased actions at thromboxane TP receptors from contractile and non-contractile prostaglandins (Xiang *et al.*, 2006), increased COX-induced oxidative stress (Goodwill *et al.*,

2008) and genetic alterations of prostacyclin synthase (Nakayama 2010). Therefore, under these conditions it is possible that the COX-derived metabolites of 2-AG also activate contractile prostaglandin receptors, blunting the vasorelaxation response, in common with other vasorelaxant mediators (reviewed in Vanhoutte *et al.*, 2009) Interestingly, this study found that patients taking NSAID, statin, and hypoglycaemic medications had significantly blunted 2-AG responses. This suggests that the vasorelaxant effect of 2-AG, and any other positive vascular consequence of 2-AG, may be compromised in patients taking these medications. However, it is difficult to determine as to whether the blunted 2-AG responses are a consequence of the medication or the disease state the medication is treating.

In conclusion, this study has shown that cannabinoids cause vasorelaxation of human mesenteric arteries often with decreased efficacy compared to animal models. The most efficacious compound tested was the highly potent CB_1 and CB_2 agonist CP55,940. This finding potentially implicates roles for these receptors in inducing vasorelaxation in the human mesenteric artery. However, given the fact that the CB_2 receptor agonist HU308 did not cause vasorelaxation, this receptor can be potentially ruled out. The most efficacious phytocannabinoid tested was the reported CB_e antagonist CBD. Finally this study has shown that the most efficacious endocannabinoid tested was 2-AG, which caused significantly greater vasorelaxation than AEA suggesting, differences between human and animal arteries.

2-AG-induced vasorelaxation was not mediated by CB_1, CB_2, TRPV1, the endothelium or metabolism by FAAH or MAGL. Rather, vasorelaxation to 2-AG in humans is dependent on COX-1 metabolism and subsequent activation of EP_4 and IP prostanoid receptors. Vasorelaxation to exogenously applied 2-AG is reduced in patients with heart disease, type-2 diabetes and patients with hypercholesterolaemia. Furthermore, 2-AG responses are also reduced in patients taking NSAIDs, statins and hypoglycaemic medications. The data from this study suggests that the vasorelaxant effects of cannabinoids are different (maximal responses and underlying mechanisms) to those observed in animal studies.

4. CHARACTERISATION OF CBD-INDUCED VASORELAXATION IN HUMAN MESENTERIC ARTERIES

4.1. Introduction

In chapter three, CBD was shown to cause vasorelaxation of human mesenteric arteries with a significantly greater R_{max} than that of THC. Few studies have examined the vasorelaxant effects of CBD in animal vasculature. Jarai and colleagues (1999) found that perfusing 10 μM CBD into phenylephrine-constricted rat mesenteric vascular beds had no effect on vascular tone. However, in arterial segments taken from rat mesenteric vascular beds, CBD caused a concentration-dependent near-maximal vasorelaxation (Offertaler *et al.,* 2003). Unfortunately, the mechanisms of action were not probed in this study. In the rat aorta, CBD (at concentrations above 100 nM) caused time-dependent vasorelaxation. This time-dependent vasorelaxation was inhibited using the PPARγ antagonist GW9662 or the SOD inhibitor diethyldithiocarbamate (DETCA) (O'Sullivan *et al.,* 2009).

CBD has been proposed as an antagonist for the putative CB_e receptor (Jarai *et al.,* 1999). In this study Abn-CBD, an analogue of CBD, which does not bind to the CB_1 receptor, caused hypotension in both $CB_1^{+/+}/CB_2^{+/+}$ and $CB_1^{-/-}/CB_2^{-/-}$ mice. These effects were inhibited by high concentrations of rimonabant, endothelium denudation and CBD (Jarai *et al.,* 1999). In humans it has been shown that Abn-CBD caused vasorelaxation of pulmonary arteries which was also inhibited by endothelial denudation, using CBD and the CBD analogue O-1918 (Kozlowska *et al.,* 2007). Interestingly, Kozlowska *et al.* (2007) observed a slight vasorelaxation effect when CBD was added to un-constricted arteries. However, this effect was not further explored. More recently, it has also been shown by the same group that CBD inhibited AEA-induced vasorelaxation of rat pulmonary arteries, which was again ascribed to antagonism of the CB_e receptor (Baranowska-Kuczko *et al.,* 2012).

To date, the effects of CBD on vascular tone *in vivo* have not been fully studied, and the limited work that has been conducted has shown conflicting findings. A recent study has shown that in pentobarbitone-anaesthetised rats, CBD causes a significant but transient 16 mmHg fall in mean arterial blood pressure without affecting heart rate (Walsh *et al.,* 2010). However, in other studies

using rats, it is reported that intraperitoneal injection of CBD has no effect on baseline heart rate or blood pressure (Resstel *et al.*, 2006; Resstel *et al.*, 2009). A recent review also concluded that chronic CBD treatment in humans fails to have any effect on blood pressure or heart rate (Bergamaschi *et al.*, 2011).

CBD has been shown to have a variety of beneficial effects in cardiovascular disease. Recent work has shown that CBD reduces endothelial and cardiac dysfunction in cardiomyopathy associated with diabetes through immune modulation and decreased oxidative stress (Rajesh *et al.*, 2007; Rajesh *et al.*, 2010). CBD reduces infarct size in a mouse model of stroke when delivered both pre- and post-ischaemic injury through activation of $5HT1_A$ receptors (Mishima *et al.*, 2005; Hayakawa *et al.*, 2007; Hayakawa *et al.*, 2007; Hayakawa *et al.*, 2008). CBD also reduces vascular inflammation associated with endotoxic shock (Ruiz-Valdepenas *et al.*, 2011), has a protective role in diabetic retinopathy (El-Remessy *et al.*, 2006) and reduces infarct size after coronary artery ligation as well as having an anti-arrhythmic effect (Walsh *et al.*, 2010).

There is a wealth of work to support the beneficial effects of CBD in the cardiovascular system, however, the direct effects of CBD on vascular tone are in doubt, with studies showing CBD causes, inhibits, or has no effect on vasorelaxation. Current work in humans has shown that CBD does not affect blood pressure when given chronically (Bergamaschi *et al.*, 2011), and in *in vitro* CBD antagonises vasorelaxation caused by other cannabinoids (Kozlowska *et al.*, 2007).

4.2. Aims

The first aim of this study was to follow on from findings in Chapter three and probe the mechanisms of actions of CBD. The second aim of this study was to investigate whether CBD caused time-dependent vasorelaxation in human mesenteric arteries, and if so was this through PPARγ mediated pathways. The third aim of this study was to analyse the arterial responses to CBD to see if, as with 2-AG, patient characteristics influenced vascular responses to CBD.

4.3. Methods

4.3.1. Patient Consent and Arterial Preparation

Informed consent was taken from patients (n=37) for the use of mesenteric arteries, only 34 patients granted the use of their personal details and medical notes in this study. Patients were receiving colorectal re-sections for cancer (n=27) and inflammatory bowel disorders (n=7) and were approached as described in section 2.2. Tissues were dissected, removing small mesenteric arteries that were either used fresh (n=28) or after overnight storage (n=9) as described in section 2.3.

4.3.2. Myography Experiments

Arteries were mounted onto a Mulvany-Halpern myograph and subject to normalisation and the standard start procedure as described in section 2.3 and 2.4. CBD concentration-response curves were carried out in viable arteries and compared to vehicle controls from an adjacent arterial segment from the same patient. To characterise CBD mechanisms of action, a range of pharmacological techniques were used as described in section 2.7, with comparisons made between responses in test and control arteries taken from the same patient.

Some arteries were incubated with antagonists and concentration-response curves were carried out to ethanol (EtOH) vehicle control. This was to assess the effects of these antagonists on the vasorelaxant effect of the vehicle control seen in Figure 3.1.

In some arteries, potential time-dependent effects of CBD were investigated. In these experiments, larger arteries (>1 mm diameter) were used, as it has previously been shown that THC causes time-dependent vasorelaxation in conduit but not resistance arteries (O'Sullivan *et al.*, 2006). Therefore, the biggest arterial segments available were taken from tissue samples and assessed for potential time-dependant effects. Artery segments were mounted on to fixed hooks and a single concentration of 10 μM CBD (in the presence or absence of 1 μM GW 9662 O'Sullivan *et al.*, 2009) or 0.1 % EtOH was added to the myograph chamber. Readings were taken every 15 minutes to assess potential time-dependent vasorelaxation.

4.3.3. *Post-hoc* Analysis

Post-hoc analysis was performed on all control CBD responses from patients who gave consent for access to medical notes (n=34). Arterial CBD responses were analysed depending on the patient's medical history and medications. Particular attention was paid to diseases and medications that have either a direct vascular impact or are vascular disease risk factors.

4.3.4. Statistical Analysis

Mean percentage relaxation is displayed with error bars representing the s.e.m, and n equalling the number of patients. Sigmoidal concentration-responses curves with a standard Hill slope of 1 were fitted to those data using GraphPad Prism. Statistical comparisons between test and control concentration-response curves and time-dependent experiments were made using 2-way ANOVA. Comparisons of pEC_{50} and R_{max} were made using 1-way ANOVA with Bonferroni's *post-hoc* test.

4.4. Results

4.4.1. Acute CBD-induced Vasorelaxation

The medical characteristics of patients used in this study are given in table 4.1. CBD caused vasorelaxation with an R_{max} of 45 ± 2% and a pEC_{50} of 5.5 ± 0.1 (Figure 4.1A & C). CBD had no effect on baseline tone (Figure 4.1B & D).

Antagonism of the CB_1 receptor using AM251 (100 nM) or LY320135 (1 μM) significantly inhibited CBD-induced vasorelaxation (Figure 4.2A & B). Antagonism of the CB_2 receptor using AM630 (100 nM) or antagonism of the proposed endothelial cannabinoid receptor using O-1918 (1 μM) had no effect on CBD-induced vasorelaxation (Figure 4.2C & D). Desensitization of the TRPV1 receptors using capsaicin (10 μM) caused inhibition of CBD-induced vasorelaxation (Figure 4.2E). CBD-induced vasorelaxation was also significantly reduced in arteries contracted using a high potassium physiological saline solution (Figure 4.2F)

Removal of the endothelium and incubation of arteries with L-NAME (300 μM) significantly altered CBD-induced vasorelaxation (Figure 4.3A & B). Incubation of the arteries with indomethacin (10 μM) had no effect on the CBD-induced vasorelaxation (Figure 4.3C).

In experiments to determine the location of the CB_1 receptor, using different arterial segments from the same patient, AM251 (100 nM) or endothelial denudation alone, slightly but not significantly reduced the pEC_{50} of CBD (Figure 4.4A & B). However, pEC_{50} values were significantly reduced in arteries that had undergone both endothelial denudation and incubation with AM251 (100 nM) (Figure 4.4A & B). R_{max} values were reduced in arteries incubated with AM251 and AM251 incubation plus endothelial denudation (Figure 4.4 A & C). However, R_{max} values were unaltered in endothelium-denuded arteries (Figure 4.4 A & C). When comparisons were made between denuded arteries incubated with AM251 and denuded arteries alone or AM251 incubated arteries alone there was no difference in pEC_{50} or R_{max} values.

The vasorelaxation to EtOH vehicle-treated control arteries (Figure 3.1D) was not inhibited by incubation with AM251, LY320135, capsaicin, L-NAME or in endothelium-denuded arterial segments (Figure 4.5 A-E). However, when EtOH was added to KPSS-contracted arteries, enhanced contraction was observed when compared to control (U46619 and endothelin-1 contracted) arteries (Figure 4.5 F).

4.4.2. Time-dependent Effects of CBD

In time-dependent vasorelaxation experiments, a single concentration of 10 μM CBD caused significant vasorelaxation compared to vehicle control (Figure 4.6A). An initial vasorelaxation of 57 ± 4 % relaxation was observed at 15 minutes, this developed to 78 ± 7 % relaxation at 120 minutes (Figure 4.6A). In the presence of the PPARγ antagonist GW 9662, this vasorelaxation was not inhibited (Figure 4.6B).

Table 4.1 Patient characteristics, diagnosis and medications CBD study

Characteristic	Range	Mean ± s.e.m
Ethnicity	34 UK white	
Male	24	
Female	10	
Age	36 – 82	65 ± 2.1
Weight (kg)	52 – 126	76 ± 3
BMI (kg/m^2)	17.5 – 36.4	27.1 ± 0.7
Vessel size (μm)	346 – 1372	701 ± 42
Bradykinin response (% reduction in pre-imposed tone)	70 – 109	85 ± 1.4
Smoking habits		
Non smokers	28	
0 – 10 CPD	3	
10 – 20 CPD	3	
Drinking habits		
< 10 units p/w	23	
10 – 20 units p/w	7	
> 20 units p/w	4	
Operation		
Right hemicolectomy	10	
Left hemicolectomy	7	
Sigmoid colectomy	5	
Anterior resection	10	
Abdominoperineal resection	1	
Total colectomy	1	
Reason for surgery		
Cancer	27	
Inflammatory bowel disorder	7	
Dukes staging		
Dukes A	10	
Dukes B	9	
Dukes C	8	
Dukes D	0	
Systolic blood pressure (mm/Hg)	110 - 188	143 ± 3
Diastolic blood pressure (mm/Hg)	62 ± 101	82 ± 1
Diabetic	10	
Heart disease	9	
Heart failure	0	
Hypercholesterolaemia	15	
Hypertensive	16	
α-1 adrenoceptor antagonist (total)	3	
Alfuzosin	1	
Terazosin	2	
ACE Inhibitors (total)	7	
Lisinopril	2	
Ramipril	5	
AT1 receptor antagonists (total)	2	
Losartan	1	
Irbesartan	1	
Beta blockers (total)	6	
Metoprolol	2	
Atenolol	4	

Characteristic	Range	Mean ± s.e.m
Calcium channel blocker (total)	3	
Amlodipine	1	
Nifedipine	1	
Lodipine	1	
Digoxin	2	
Diuretics (total)	3	
Furosemide	3	
GTN	3	
Hypoglycaemic Medication (total)	6	
Gliclazide	3	
Metformin	3	
NSAID Medication	14	
Aspirin	8	
Ibuprofen	2	
Paracetamol	7	
Co Codamol	1	
Statin (total)	14	
Atorvastatin	4	
Simvastatin	9	
Pravastatin	1	
Thiazolidinedione (total)	1	
Pioglitazone	1	

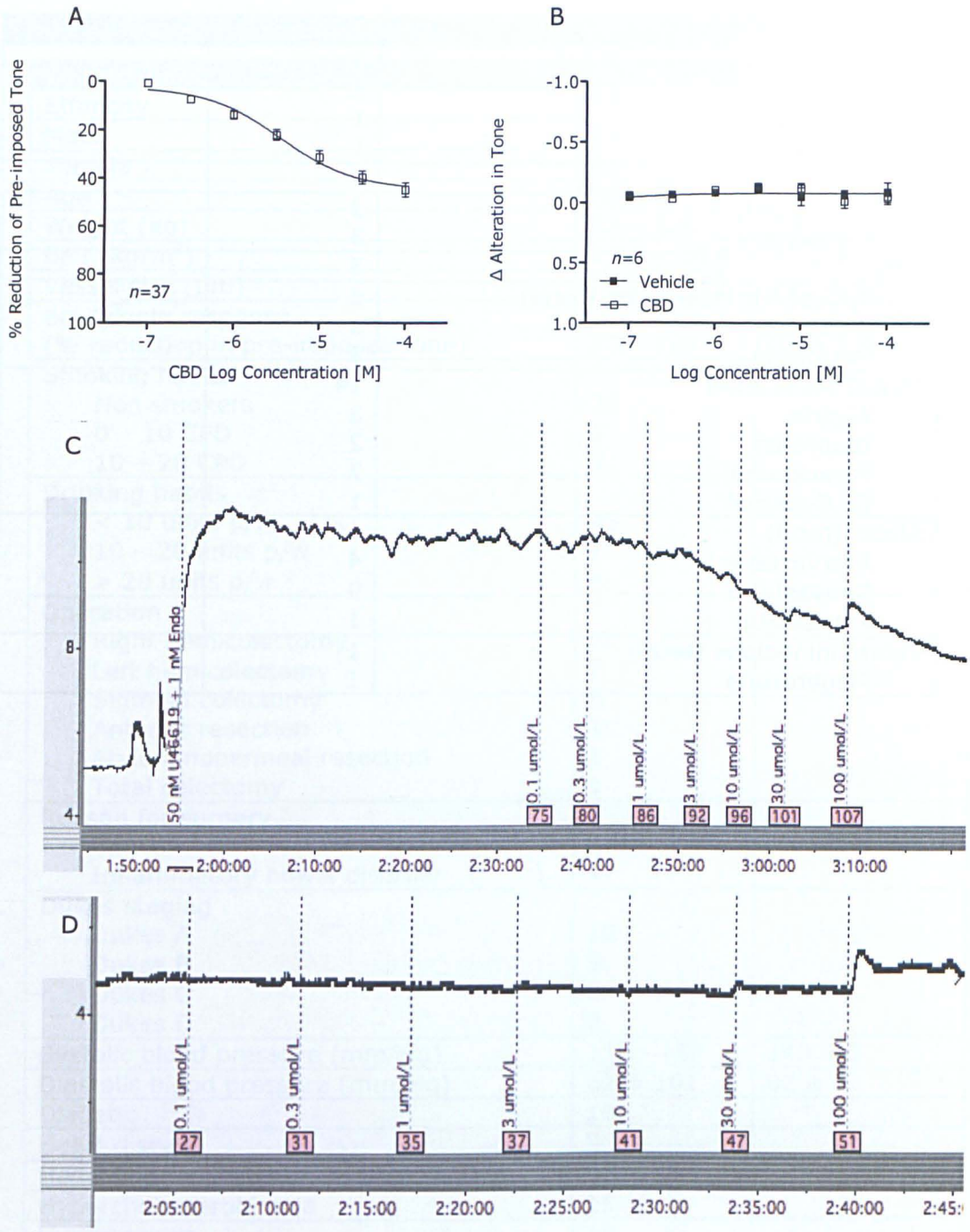

Figure 4.1 CBD responses
CBD-induced vasorelaxation in all patient samples with representative trace (A and C). Effects of CBD on baseline compared to vehicle control and representative trace (B and D). Comparisons made using 2-way ANOVA.

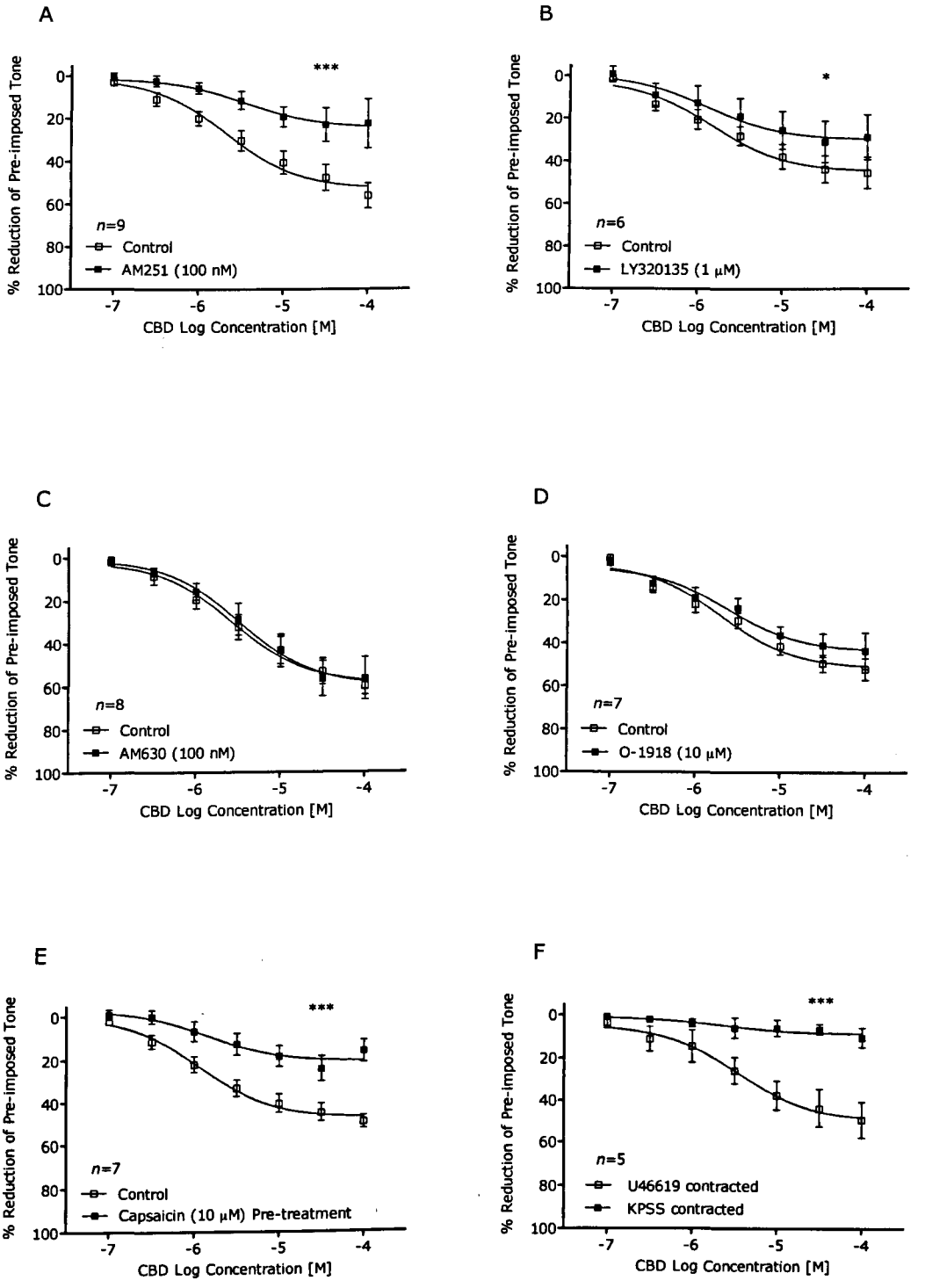

Figure 4.2 Potential receptor and ion channel involvement in CBD responses

CBD-induced vasorelaxation in arteries incubated with the CB_1 antagonist AM251 (A), the CB_1 antagonist LY320135 (B), CB_2 antagonist AM630 (C), the putative CB_e receptor antagonist O-1918 (D), in arteries where the TRPV1 receptor had been desensitised after 1-hour incubation with the TRPV1 agonist capsaicin (E) or in arteries contracted with high potassium physiological salt solution (F). Comparisons made using 2-way ANOVA, * $P<0.05$ and *** $P<0.001$.

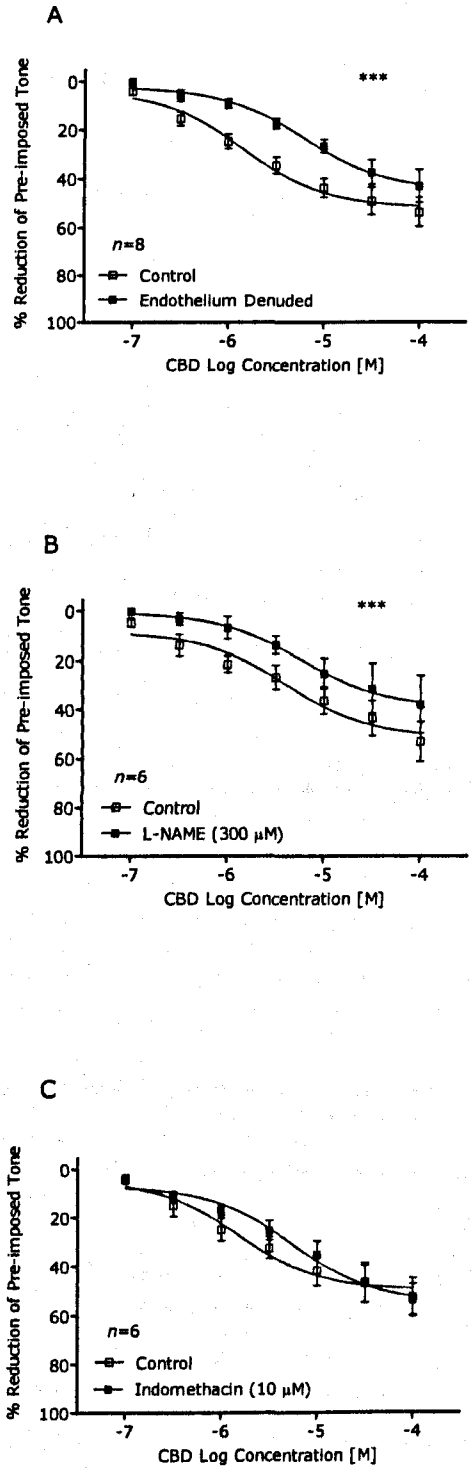

Figure 4.3 Potential involvement of the endothelium and COX in CBD responses

CBD-induced vasorelaxation after endothelium denudation (A), in arteries that have been incubated with the nitric oxide synthase inhibitor L-NAME (B) or in arteries incubated with the non-selective COX inhibitor indomethacin (C). Comparisons made using 2-way ANOVA, *** $P<0.001$.

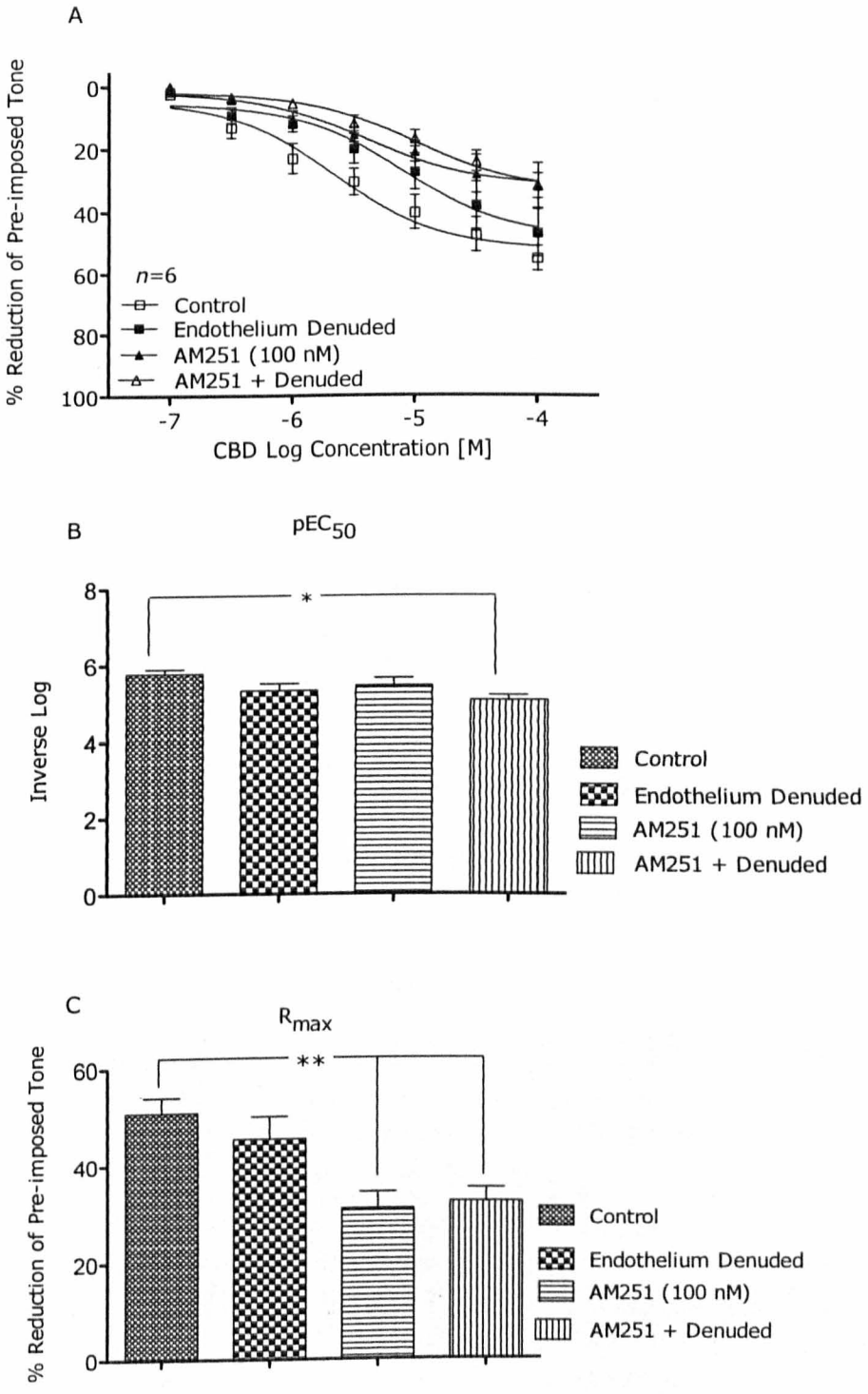

Figure 4.4 Experiments to establish the potential relationship between the CB_1 receptor and the endothelium.
CBD-induced vasorelaxation in control arteries (empty squares), endothelium-denuded arteries (filled squares), in arteries incubated with the CB_1 antagonist AM251 (filled triangles) or in arteries that are endothelium-denuded and incubated with AM251 (empty triangles)(A). Comparisons between pEC_{50} (B) and R_{max} (C), values derived from (A) Comparisons made using 1-way ANOVA and Bonferroni *post-hoc* test. **P<0.05 and ** P<0.01.

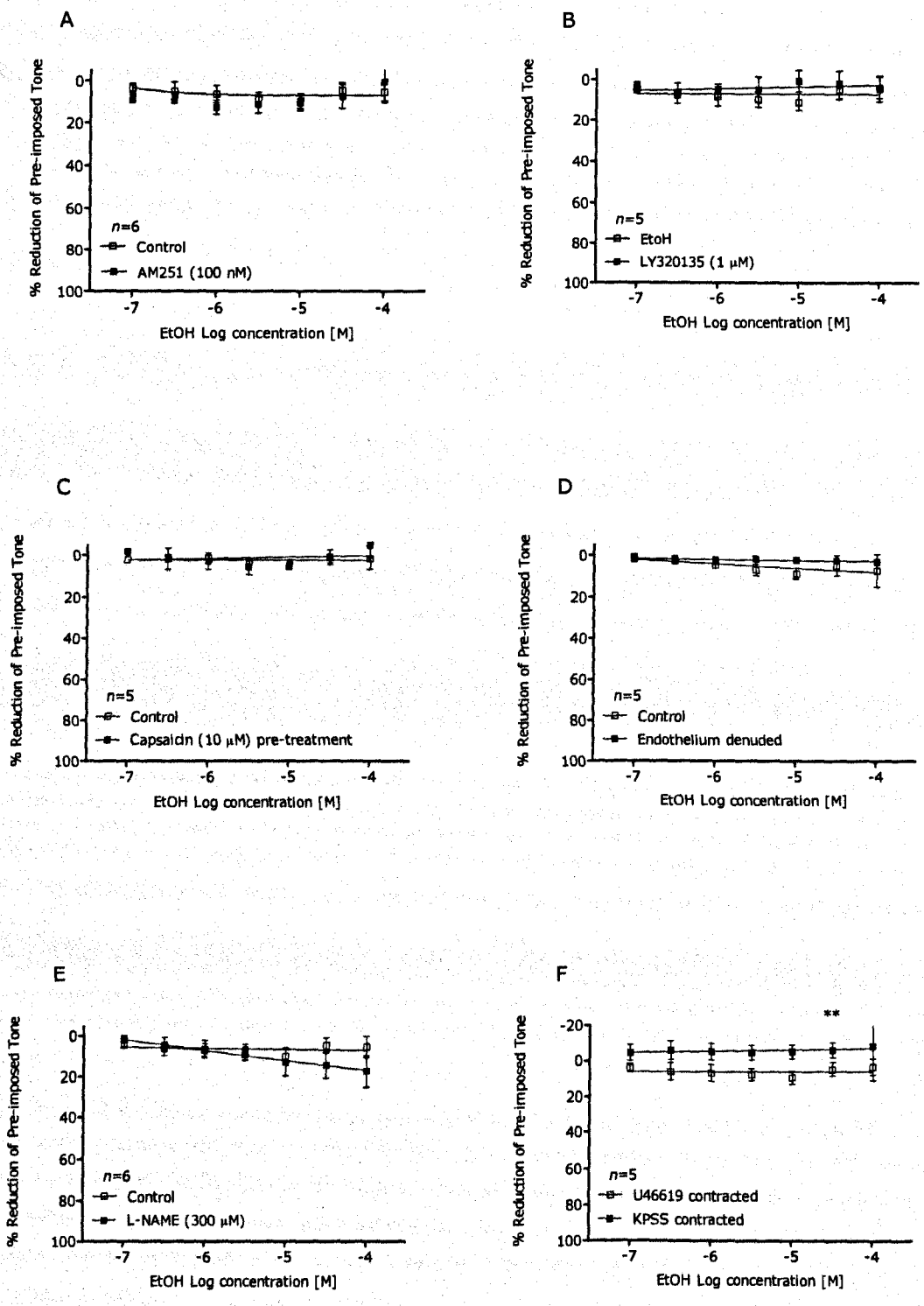

Figure 4.5 Ethanol concentration-response curves.
Ethanol concentration-response curves carried out in arteries under the following conditions; incubation with 100 nM AM251 (A), incubation with 1 μM LY320135 (B), 1 hour incubation with capsaicin (C), after endothelium-denudation (D), in the presence of L-NAME or in arteries contracted using KPSS. Comparisons made using 2-way ANOVA. ** $P<0.01$.

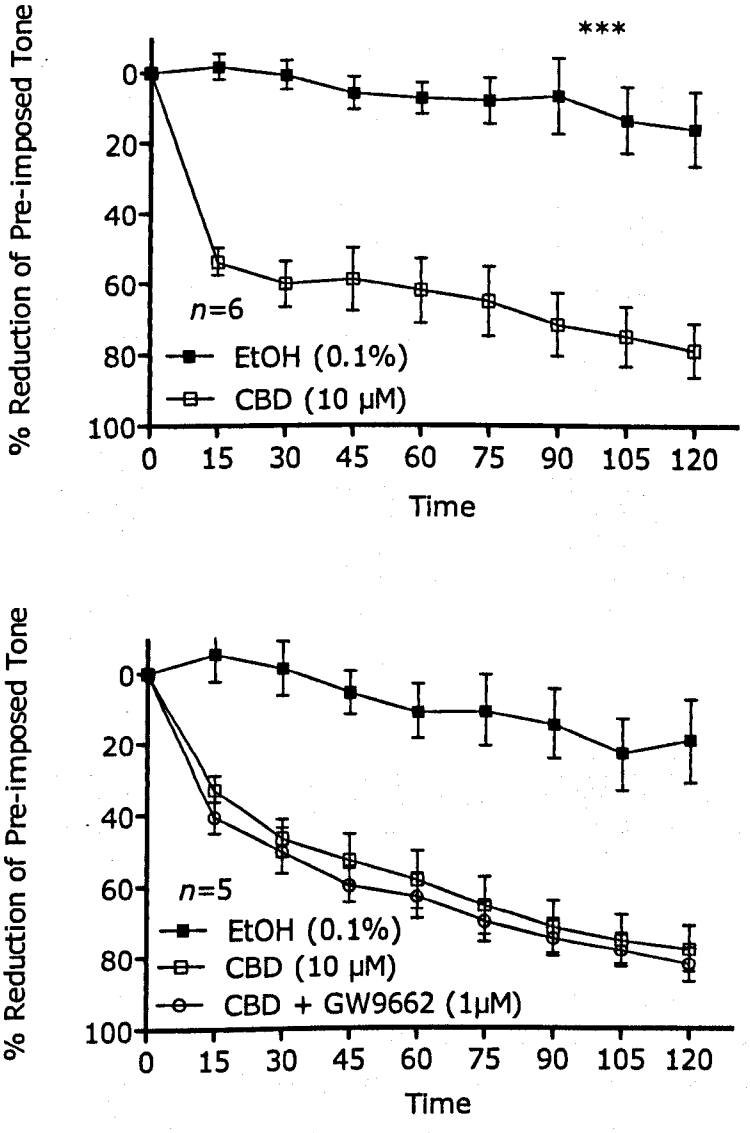

Figure 4.6 Potential time-dependent effects of CBD
Time-dependent vasorelaxation to a single concentration of CBD (10 μM) compared to ethanol vehicle control (A) and in the presence of the PPARγ antagonist GW 9662 (B). Comparisons made using 2-way ANOVA. $P<0.001$ ***.

4.4.3. *Post-hoc* Analysis of Patient Medical Notes

Due to the variability of control responses to CBD among patients (response range at 100 μM CBD, 2.2 – 75% reduction in pre-imposed tone), and that 2-AG responses are reduced depending on patient medical condition or medication, *post-hoc* analysis of patient vasorelaxant responses to CBD was carried out. The variables compared were diagnosis, current medication and cardiovascular risk factors. CBD responses were reduced in males compared to females and positively correlated with bradykinin response (Figure 4.7A & D). CBD responses did not correlate with age, nor were they reduced in patients with cancer and those without (Figure 4.7B & C). CBD responses were significantly reduced in patients diagnosed with hypercholesterolaemia, type-2 diabetes and hypertension (Figure 4.8B, C & D). Interestingly, analysis of patient notes showed a modest yet significant increase in CBD-induced vasorelaxation in patients that smoke compared to those that do not (Figure 4.8F). CBD-induced vasorelaxation was not reduced in patients with heart disease or a BMI >25 kg/m^2 (Figure 4.8 A & E). CBD responses were reduced in those taking NSAIDs, statins, hypoglycaemic medication and beta-blockers (Figure 4.9A - D), but not those taking ACE inhibitors (Figure 4.9E).

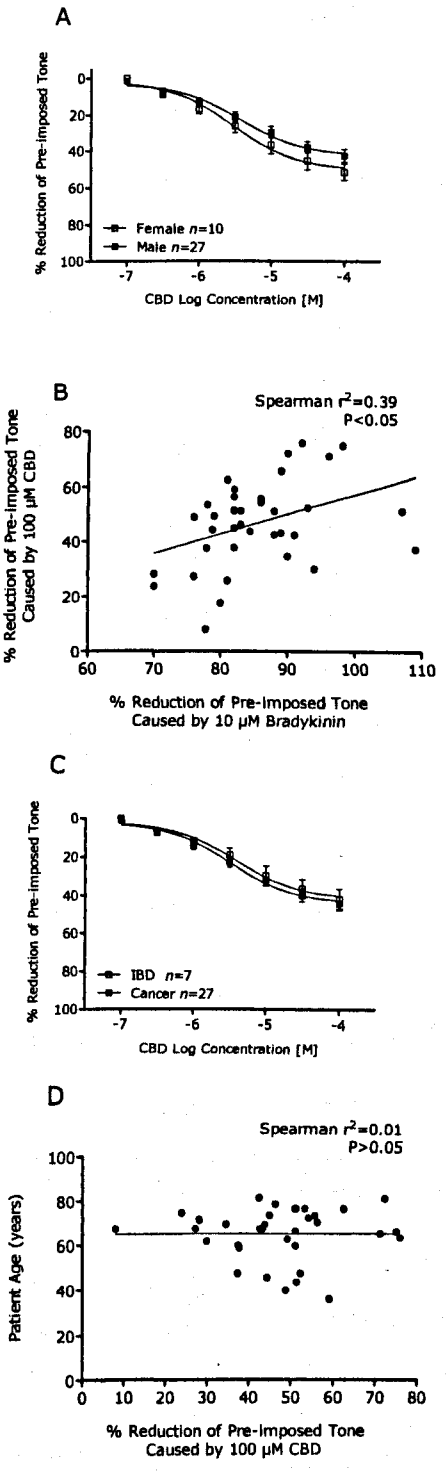

Figure 4.7 Patient characteristics and CBD responses
CBD-induced vasorelaxation in females compared to males (A). CBD R_{max} responses did not correlate with patient age (B). CBD-induced vasorelaxation in patients with cancer compared to patients without cancer (C). CBD R_{max} responses correlated with bradykinin response from the same artery (D). Comparisons made using 2-way ANOVA and correlations made using Spearman correlation coefficient. * $P<0.05$.

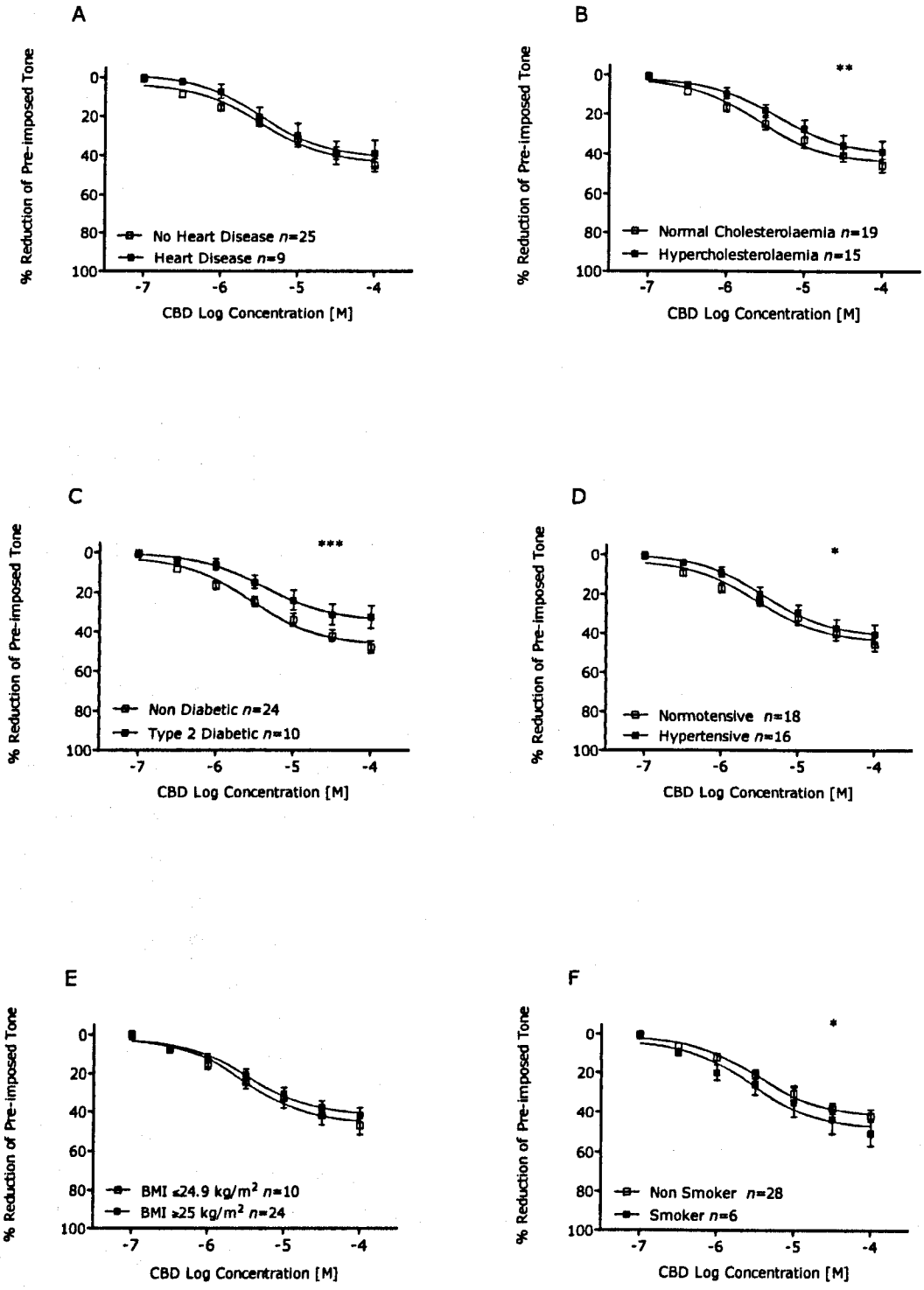

Figure 4.8 Cardiovascular disease/disease risk factors and CBD responses

CBD concentration-response curves in patients with heart disease (A), hypercholesterolaemia (B), type-2 diabetes (C), hypertension (D), elevated BMI (E) and in patients who smoke (F). Comparisons were made between patients with a given characteristic and those without using 2-way ANOVA, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

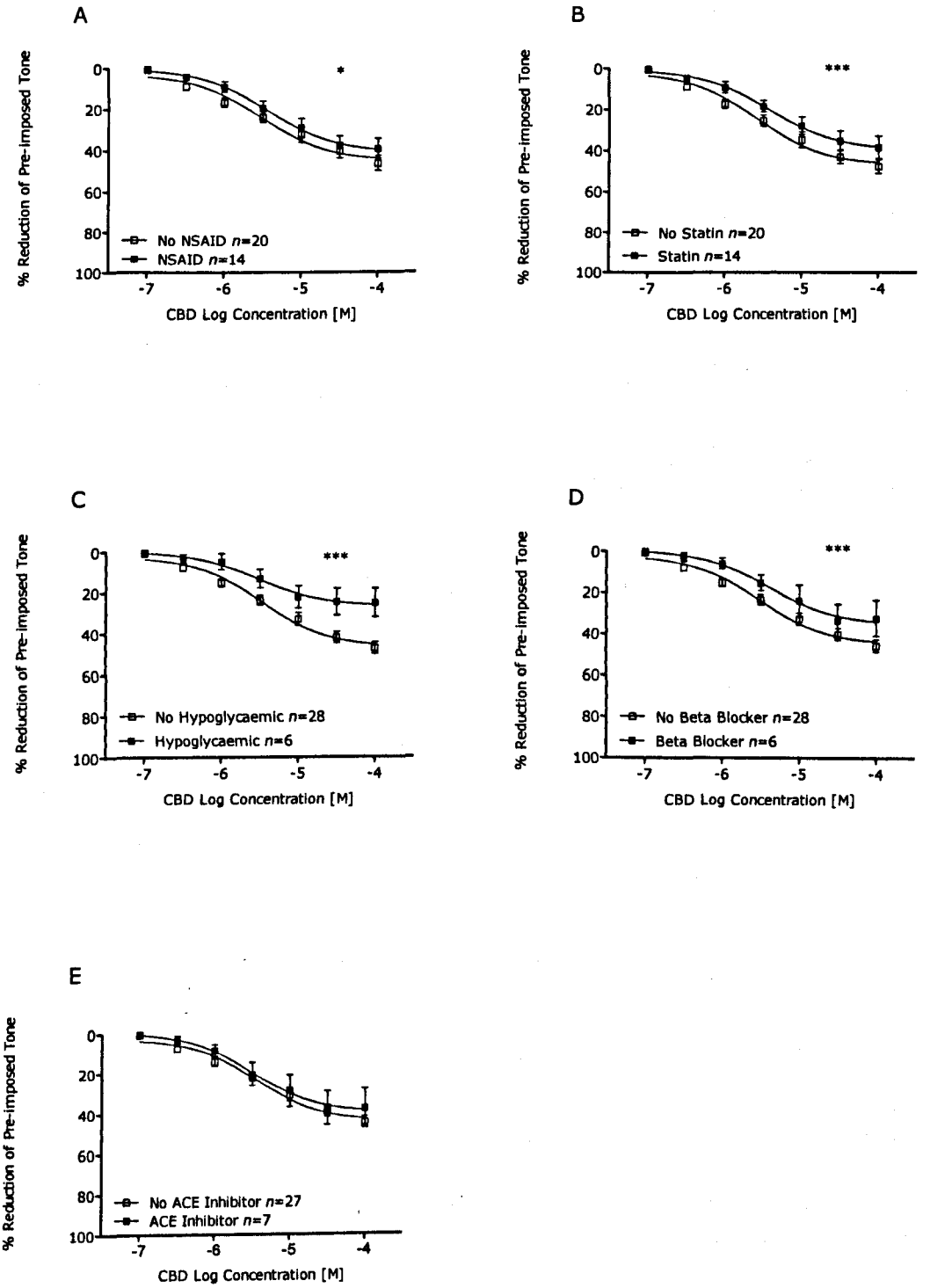

Figure 4.9 Patient medication and CBD responses
CBD concentration-response curves in patients taking NSAIDs (A), statins (B), hypoglycaemic (C), beta-blocking (D) and ACE inhibiting medication (E). Comparisons were made between patients taking a given medication and those that were not using 2-way ANOVA. * $P<0.05$, and *** $P<0.001$.

4.5. Discussion

The aims of this chapter were to characterise the vasorelaxant effects of CBD in human mesenteric arteries, to assess CBD for potential time-dependent PPARγ-mediated vasorelaxation and to assess CBD control responses in light of the patient medical history. This study has shown that CBD-induced vasorelaxation is dependent on CB_1 and TRPV1 receptor activation, the endothelium, NO and ion channel modulation. In human small resistance mesenteric arteries, CBD does not cause PPARγ-mediated vasorelaxation. *Post-hoc* analysis of patient responses revealed slight, but significant reductions in responses to CBD in patients with hypercholesterolaemia, type-2 diabetes and hypertension.

In human mesenteric arteries CBD caused half-maximal vasorelaxation of human mesenteric arteries with a pEC_{50} in the mid micromolar range. These findings are similar with those reported in the rat mesenteric artery, where CBD causes vasorelaxation with similar potency to that observed in this study, however, in the rat model CBD caused near maximal vasorelaxation (Offertaler *et al.,* 2003). This might suggest that the efficacy of CBD is reduced in human vasculature, which is in contrast to other work which has shown that cannabinoid potency, but not efficacy, is reduced when comparing human and rat vascular responses to cannabinoids (Kozlowska *et al.,* 2007; Kozlowska *et al.,* 2008). This finding could be explained by species differences which have previously been shown with other cannabinoids (Randall *et al.,* 2004). However, an alternative explanation could lie in the patients from whom samples were taken, as less than half the patient population used in this study were without co-morbidities that *post-hoc* analysis showed reduced CBD responses. Hypertension, diabetes, increased cholesterol and smoking were observed to reduce CBD responses and are associated with increased reactive oxygen species, increased vasoconstrictor prostanoids, decreased vasorelaxant responses and subsequent endothelial and vascular smooth muscle dysfunction (Vanhoutte *et al.,* 2009). It is also possible that differences in the efficacy could be explained by the differences in contractile agent used. Offertaler and colleagues (2003) contracted arteries using phenylephrine, however in this study U46619 and endothelin-1 were used. In rat mesenteric arteries and human

coronary myocytes, U46619 has been shown to inhibit BK_{Ca} channels and SK_{Ca} channels (Crane and Garland 2004; Li *et al.*, 2010). Potassium channel activation was found to be a potential contributor to CBD-induced vasorelaxation in human mesenteric arteries. Therefore, BK_{Ca} and SK_{Ca} channel inhibition caused by U46619 could explain the reduced efficacy seen in this work.

In animal models, activation of CB_1, CB_2 and TRPV1 receptor sites has been implicated in cannabinoid-induced vasorelaxation (Zygmunt *et al.*, 1999; O'Sullivan *et al.*, 2004; O'Sullivan *et al.*, 2005). Since human vascular smooth muscle and endothelial cells express these receptors (Sugiura *et al.*, 1998; Liu *et al.*, 2000; Fantozzi *et al.*, 2003; Rajesh *et al.*, 2007; Rajesh *et al.*, 2008; Wang *et al.*, 2008), and CBD has been shown to bind to these receptors at low micromolar concentrations (Showalter *et al.*, 1996; Bisogno *et al.*, 2001), they were considered potential mechanisms that underpinned CBD-induced vasorelaxation. Antagonism of the CB_1 receptor using AM251 revealed inhibition of CBD-induced vasorelaxation. Since CBD has a complex pharmacology at the CB_1 receptor, whereby it has been shown to cause antagonism/inverse agonism at micromolar concentrations (reviewed Pertwee 2008) and AM251 is reported to have effects at targets other than CB_1 (reviewed Pertwee 2005) including activation of GPR55 (Ryberg *et al.*, 2007), the role of CB_1 was confirmed using another antagonist at this receptor, LY320135 which is a CB_1 antagonist that is structurally distinct from AM251 and rimonabant (Felder *et al.*, 1998). LY320135 also showed inhibition of CBD-induced vasorelaxation, albeit less inhibition than observed using AM251, confirming the role for CB_1 in CBD-induced vasorelaxation. However given that in various cell types, CBD is reported to be void of activation of cannabinoid receptors (Bisogno *et al.*, 2001; Lauckner *et al.*, 2005), an alternative explanation for the inhibitory effects of AM251 in CBD-induced vasorelaxation may lay in the pharmacology of this antagonist. AM251, in an invertebrate model void of cannabinoid receptors, has been shown to reverse capsaicin induced long term synaptic depression (Yuan and Burrell 2010), suggesting that AM251 may inhibit TRPV1 responses. The plausibility of this explanation is increased considering that TRPV1 desensitisation also inhibited CBD-induced vasorelaxation. However, it is unknown if LY320135 has activity at TRPV1. Therefore a further explanation may be that CBD acts as an inverse agonist at CB_1

(reviewed Pertwee 2008), and as CB_1 receptors are negatively coupled to cAMP resulting in inhibition of PKA, inverse agonism would increase PKA activity. Elevations of PKA are reported to increase the sensitivity of TRPV1 receptors (Di Marzo *et al.,* 2002). Therefore, as this study also shows that TRPV1 desensitisation inhibits CBD-induced vasorelaxation it is possible that CBD does not directly cause vasorelaxation through the CB_1 receptor. Instead there is the possibility that CBD sensitises the TRPV1 receptor through inverse agonism of the CB_1 receptor. However, it has recently been shown that CBD decreases gut permeability in a CB_1-dependent manner that is not dependent on TRPV1 (Alhamoruni *et al.,* 2010). Alhamoruni *et al.* (2010) suggest direct activation of CB_1 by CBD in the gut, therefore the possibility of CBD directly activating CB_1 in the current study cannot be ruled out.

Antagonism of the CB_2 receptor using AM630 did not inhibit CBD-induced vasorelaxation. Other studies have also shown that CB_2 receptor activation is not commonly found to underpin the vasorelaxant effects of cannabinoids (White *et al.,* 2001; Hoi and Hiley 2006; O'Sullivan *et al.,* 2009). This finding is further supported in Chapter Three, as the selective CB_2 agonist HU308 did not cause vasorelaxation.

Desensitisation of TRPV1 channels using the TRPV1 agonist capsaicin also caused inhibition of CBD-induced vasorelaxation. Work in the rat mesenteric artery has shown that vasorelaxation to two chemically closely-related cannabinoids, THC and cannabinol, is inhibited by capsaicin pre-treatment (Zygmunt *et al.,* 2002). Plant-derived cannabinoids are good activators of the TRPV channel family (De Petrocellis *et al.,* 2011), and CBD has been suggested to have a therapeutic use in cancer cell apoptosis (Yamada *et al.,* 2010), reduction of cancer cell invasion (Ramer *et al.,* 2010), anti-hyperalgaesic responses to both chronic and acute inflammatory pain (Costa *et al.,* 2004; Costa *et al.,* 2007) through activation of TRPV channels. Furthermore, in cultured rat dorsal root ganglion, CBD, through TRPV2, causes release of calcitonin gene-related peptide (CGRP), a known vasomodulator associated with cannabinoid/TRPV channel induced vasorelaxation (Qin *et al.,* 2008). Therefore, it would be of future interest to probe the potential involvement of other TRPV receptors using the non-selective TRPV channel blocker ruthenium red and or selective channel blockers.

Taken together, the above findings show that CBD causes vasorelaxation that is sensitive to CB_1 receptor antagonism and TRPV1 receptor desensitisation. Given the reported pharmacology between the CB_1 receptor and CBD, the role for the CB_1 receptor, and potential regulation of the TRPV1 receptor, requires further investigation. Also, the above findings taken together with Chapter Three would further suggest that the CB_2 receptor does not mediate vasorelaxation in human mesenteric arteries.

The endothelium has been shown to mediate vasorelaxation of the CBD analogue Abn-CBD, and this vasorelaxation is often associated with activation of the putative CB_e receptor that can be antagonised using O-1918 (Begg *et al.,* 2003; Offertaler *et al.,* 2003; Kozlowska *et al.,* 2007). The present study found that removal of the endothelium reduced responses to CBD in submaximal concentration ranges. However, in the presence of O-1918, CBD-induced vasorelaxation is unaltered, suggesting that the endothelial component is not linked to activation of the as yet unidentified 'endothelial' receptor. NO is a well-known endothelium-dependent vasorelaxant and has been shown to mediate vasorelaxation to anandamide and WIN 55,212-2 in rat renal vasculature and rat aortae respectively (Deutsch *et al.,* 1997; Dannert *et al.,* 2007). Incubation with the nitric oxide synthase inhibitor L-NAME reduced CBD-induced vasorelaxation at sub-maximal concentrations but not at maximal concentrations, in a similar manner to that observed in arteries that had been endothelium-denuded.

This suggests that CBD acts on the endothelium and releases nitric oxide. Previous studies have shown AEA can increase NO through CB_1- (Deutsch *et al.,* 1997) and TRPV1- (Poblete *et al.,* 2005) mediated pathways, therefore the NO component of CBD-induced vasorelaxation might in part be through receptor-mediated NO release.

In rabbit arterioles the effects of both AEA and THC are inhibited by indomethacin (Ellis *et al.,* 1995), suggesting the role of COX metabolism in vasorelaxation to both endogenous cannabinoids and phytocannabinoids. Therefore, the potential for endothelial or smooth muscle production of COX and subsequent metabolism of CBD into vasoactive compounds was investigated. Indomethacin had no effect on CBD-induced vasorelaxation, suggesting COX metabolism of CBD was not involved in vasorelaxation. It has recently been shown however,

that CBD attenuates inducible COX expression in human colon adenocarcinoma cells (Ruhaak *et al.,* 2011).

Cannabinoid-induced vasorelaxation has previously been shown to be inhibited in arteries pre-contracted with high potassium solution (White and Hiley 1998). The present study also reports that CBD-induced vasorelaxation is inhibited in arteries contracted using high potassium solution. Contractions to high potassium solution are mediated through influx of calcium through voltage-operated calcium channels (Karaki *et al.,* 1997). Therefore, the inability of CBD to cause vasorelaxation might suggest that CBD is unable to inhibit calcium influx through these channels. However, increases in extracellular potassium have been shown to inhibit potassium channel-mediated hyperpolarisation (reviewed in Quignard *et al.,* 1999), therefore, a predominant mechanism of CBD-induced vasorelaxation might be activation of potassium channels and subsequent hyperpolarisation.

The CB_1 receptor is expressed in both human endothelial cells and vascular smooth muscle cells in culture (Sugiura *et al.,* 1998; Liu *et al.,* 2000). Further experiments were carried out to establish if the endothelium sensitive and AM251 sensitive components were additive, and therefore potentially suggesting the location of the CB_1 receptor. To test this arterial segments from the same patient were either untreated (control), endothelium denuded, incubated with AM251 or endothelium denuded and incubated with AM251. Comparisons of CBD pEC_{50} and R_{max} values were then made between differing treatments. This revealed that there were no differences in pEC_{50} values between untreated arteries and arteries that were endothelium denuded alone or AM251 incubated alone. However, there was a significant difference between untreated arteries and endothelium denuded AM251 incubated arteries, suggesting an additive effect. When R_{max} comparisons were made there were significant ($P<0.01$) reductions of 20% (AM251 incubated) and 18% (endothelium denuded AM251 incubated) in R_{max} compared to untreated arteries. However, when AM251 incubated arteries and endothelium denuded AM251 incubated arteries were compared to endothelium denuded alone there was a 14% and 13% reduction in R_{max} respectively. These differences were not significant, although they show a trend towards being significantly different to endothelium denuded arteries alone. Therefore these experiments were inconclusive in showing whether the endothelium dependent and

AM251 sensitive components were additive, and further experiments are needed to draw a satisfactory conclusion.

In rat aortae, CBD caused time-dependent vasorelaxation that can be inhibited by PPARγ antagonism (O'Sullivan *et al.*, 2009). Although this study reports that, over time, CBD-induced vasorelaxation gradually increased, this effect was not inhibited by PPARγ antagonism. THC caused similar PPARγ-mediated time-dependent vasorelaxation in the rat aorta (O'Sullivan *et al.*, 2005). However, in third order mesenteric arteries THC was unable to cause time-dependent vasorelaxation that is significantly different from vehicle control (O'Sullivan *et al.*, 2006). The PPARγ receptor is expressed in human aortic and coronary smooth muscle cells (Benson *et al.*, 2000), human saphenous vein endothelial cells (Marx *et al.*, 1999) and in human inferior mesenteric arteries (Soumian *et al.*, 2005). Furthermore, it has been shown that incubation with a PPARγ agonist (GW1929) can enhance endothelium-dependent vasorelaxation in human omental arteries from elderly patients or patients with cardiovascular disease risk factors (Angulo *et al.*, 2012). Therefore, the lack of PPARγ-mediated vasorelaxation seen to CBD may be due to vascular bed or arterial size differences. However, an interesting observation was the non-recoverable vasorelaxation observed to CBD. In rat G3 mesenteric arteries THC did not cause PPARγ mediated vasorelaxation and the vasorelaxation to THC started to recover during the 2 hour period (O'Sullivan *et al.*, 2006). The present study suggests that the non-recoverable response to CBD was mediated by either the CB_1 and TRPV1 receptors, as seen in acute vasorelaxation, or by an additional unknown mechanism. CB_1 and TRPV1 receptors are both prone to desensitization and internalisation (Sim *et al.*, 1996; Zygmunt *et al.*, 1999). Therefore, if these receptors mediated the effects of CBD over 2 hours it would suggest that 10 μM CBD over a 2 hour period did not desensitise these receptors in this system.

Taken together these findings show that CBD-induces vasorelaxation of the human mesenteric artery dependent on CB_1 and TRPV1 receptor activation, the endothelium, NO and ion channel modulation. This study also shows there is a trend for increased inhibition in CBD response when arteries are denuded of their endothelium and incubated with AM251 compared to endothelium denuded alone. Finally, mechanistic work has shown that CBD causes

vasorelaxation over a 2-hour period that is not mediated by the PPARγ receptor.

Given the variability of the responses seen to CBD and the influence of patient medical conditions and medications seen in chapter three, *post-hoc* analysis of patient medical notes was undertaken.

This study found that CBD-induced vasorelaxation was enhanced in females compared to males. The enhanced vasorelaxation observed in female patient arteries compared to males may be due to protective effects of oestrogen on endothelial function (Novella *et al.,* 2012). However, it is worth noting that early research also showed that CB_1 receptor expression was increased in the leukocyte cells of females when compared to males (Onaivi *et al.,* 1999).

CBD responses were not dependent on patient age. Increasing age is associated with endothelial dysfunction (Vanhoutte *et al.,* 2009), yet CBD responses did not correlate with age in this study which may suggest that mechanisms behind CBD-induced vasorelaxation may be preserved in aging populations.

CBD responses were not different between cancer patients and patients with inflammatory bowel disorders. Inflammatory bowel disorders are associated with increased COX-2 expression (Tabernero *et al.,* 2003). However, the effects of CBD were unaltered by COX inhibition, suggested COX levels have no bearing on CBD–induced vasorelaxation.

Positive correlations were observed between CBD and bradykinin response. In humans mesenteric arteries, as in animals, bradykinin causes vasorelaxation that is dependent on the endothelium (Cherry *et al.,* 1982). This finding adds further support to the role of the endothelium in CBD-induced vasorelaxation.

Similar to 2-AG, CBD responses are reduced in the vasculature of those with some cardiovascular diseases. CBD responses were reduced in those with increased cholesterol, diabetes and hypertension. Key mechanisms underlying CBD-induced vasorelaxation were CB_1 and the TRPV1 receptor activation. CB_1 receptor expression is increased in vascular diseases such as atherosclerosis and acute myocardial infarction (Sugamura *et al.,* 2009; Wang *et al.,* 2011). However, in rats fed high cholesterol diets CB_1 receptor expression is reduced (Hayakawa *et al.,* 2007). Also, in diabetes TRPV1 receptor coupling to nitric oxide and BK_{Ca} potassium channels is disrupted (Guarini *et al.,*

2012). Therefore, it is likely that the decreased responses of CBD in patients with cardiovascular disease are a result of complex changes occurring at a receptor or post-receptor level, whereby receptor expression potentially increases to compensate for loss of function or is down-regulated. However, it should be noted that the reductions observed ranged from 3% in patients with hypertension to 12% in type two diabetic patients. Whilst these values were significant they may have little *in vivo* significance on systemic blood pressure. Wheal *et al.* (2007) reported that *in vivo* administration of AEA to conscious rats caused a 40% decrease in mesenteric conductance that was in parallel to a 20% increase in mean arterial pressure. Wheal *et al.* (2007) in the same study, also reported that AEA caused both increases and decreases in vascular conductance depending on the vascular bed studied. Therefore, in isolated arteries, small reductions in vasorelaxation should be interpreted with caution, as such effects may not be mirrored in other vascular beds and may not translate to *in vivo* conditions.

CBD responses are reduced in patients taking NSAID, statin, hypoglycaemic and beta blocking medication. In some patients it has been reported that NSAID medication, through loss of PGI_2, increases the risk of cardiovascular and vascular disease (Amer *et al.,* 2010). However, statins and hypoglycaemic agents including simvastatin and metformin have been shown to improve endothelial function through reduced thromboxane activities (McNeish *et al.,* 2012; Puyo *et al.,* 2012). Therefore, with the potential exception of NSAIDs, it is likely that the reduced vasorelaxation observed to CBD is a result of the underlying medical condition and not the medication.

In conclusion, this study reports that CBD causes vasorelaxation of the human mesenteric artery. This vasorelaxation is mediated through CB_1, TRPV1, the endothelium, nitric oxide and potassium hyperpolarisation. CBD also causes time-dependent vasorelaxation of human mesenteric arteries, but this was not due to PPARγ activation. The vasorelaxant effects of CBD are reduced in patients with hypercholesterolaemia, type-2 diabetes and hypertension. Given that CBD partly causes vasorelaxation through CB_1 and TRPV1 receptors, and that these receptors are altered in diseases that affect the vasculature, further

work is needed to fully understand alterations in these receptors and how they interact with endocannabinoids.

5. CHARACTERISATION OF AEA-INDUCED VASORELAXATION IN HUMAN MESENTERIC ARTERIES

5.1. Introduction

AEA causes maximal vasorelaxation of third order rat mesenteric arteries with potency in the micromolar range (Randall *et al.*, 1997; White and Hiley 1997; O'Sullivan *et al.*, 2004). In rat third order mesenteric arteries, vasorelaxation to AEA is underpinned by several key pathways including CB_1 and CB_2 receptor activation (Randall *et al.*, 1997; White and Hiley 1997; O'Sullivan *et al.*, 2004), liberation of EDHF, activation of TRPV1 channels (O'Sullivan *et al.*, 2004) and activation of the putative CB_e receptor (Offertaler *et al.*, 2003). Whilst there are some mechanistic similarities between different vascular beds/species, some differences have been observed. For example, inhibition of AEA-induced vasorelaxation by inhibiting nitric oxide synthase has only been observed in rat small renal arterioles (Deutsch *et al.*, 1997), but not in rat mesenteric arteries (O'Sullivan *et al.*, 2004). Also, AEA induces 25-50% vasorelaxation of rabbit cerebral arterioles (Ellis *et al.*, 1995), 50% vasorelaxation of bovine coronary arteries (Pratt *et al.*, 1998), and 80% vasorelaxation of ovine coronary arteries (Grainger and Boachie-Ansah 2001) through COX-dependent pathways (Ellis *et al.*, 1995; Pratt *et al.*, 1998; Grainger and Boachie-Ansah 2001). However, in rat mesenteric arteries, metabolism of AEA has been suggested to limit the vasorelaxant effects of AEA (Ho and Randall 2007). These authors showed that incubation of third order rat mesenteric arteries with COX or FAAH inhibitors was able to significantly increase the potency of AEA. The study concluded that in third order rat mesenteric arteries the endothelium might represent a metabolic barrier limiting AEA-induced vasorelaxation (Ho and Randall 2007).

Taken together these studies show that the efficacy and mechanisms of AEA-induced vasorelaxation differ depending on the species and vascular bed studied.

In the rat aorta, AEA causes acute vasorelaxation that is a quarter of the amount seen in rat third order mesenteric arteries (O'Sullivan *et al.*, 2005). However, prolonged exposure to AEA (5 μM) causes time-dependent vasorelaxation of around 50% over a 2-hour period (O'Sullivan *et al.*, 2004; O'Sullivan *et al.*, 2009). Vasorelaxation

is inhibited by PPARγ antagonism (GW9662), inhibition of protein synthesis (cycloheximide), endothelial denudation, nitric oxide inhibition (L-NAME) and superoxide dismutase inhibition (diethyldithiocarbamate) (O'Sullivan *et al.,* 2009). Metabolism of AEA through FAAH was not involved in the PPARγ-mediated actions of AEA (O'Sullivan *et al.,* 2009).

In humans, plasma AEA concentrations are reported to be between 0.3-2.5 nM (Zoerner *et al.,* 2009). Circulating AEA concentrations are raised in a variety of disease states that directly affect the vasculature or in patients that have risk factors for cardiovascular disease. For example, AEA levels are increased by 35% in obese patients plasma (Engeli *et al.,* 2005), increased by 65% in blood serum from patients with type-2 diabetes compared to healthy controls (Matias *et al.,* 2006) or increased by 200% in tissue compared to obese non-type-2 diabetic patients (Annuzzi *et al.,* 2010), correlate with coronary dysfunction (Quercioli *et al.,* 2011) and increased by 55% in semi purified plasma samples from patients with portal hypertension associated with cirrhosis (Fernandez-Rodriguez *et al.,* 2004). Interestingly, the latter study reported that elevations in AEA concentrations were not associated with any haemodynamic changes (Fernandez-Rodriguez *et al.,* 2004). The reasons behind the increase in AEA concentrations in these conditions are unknown. Animal studies might suggest that the increase in AEA serves as a protective measure against further vascular decline. *In vivo* work has shown that the systemic hypotensive effect of AEA is apparent in SHR but not normotensive rats (Lake *et al.,* 1997; Wheal *et al.,* 2007). *In vitro* work supports this showing that increased AEA and increased CB_1 levels are associated with decreased arterial contractions to high potassium and phenylephrine, with enhanced vasorelaxant responses to ACh and SNP in the mesenteric arteries of biliary cirrhotic rats (Yang *et al.,* 2007). However, there may be species and vascular bed differences in this finding as Wheal *et al.* (2009) found that, in the mesenteric vascular bed, AEA caused less vasorelaxation in arteries of hypertensive rats compared to normotensive controls. However, in the aortae of the same rat model AEA was shown to have increased efficacy (Wheal and Randall 2009).

In humans there has been limited work looking at the vascular effects of AEA. In human arteries, AEA is ineffective as a vasorelaxant

in myometrial arteries (Kenny *et al.,* 2002). However, AEA causes increased blood flow in the forearm circulation when applied topically but not intra-arterially (Movahed *et al.,* 2005). In this study Movahed *et al.* (2005) show that the vasorelaxation caused by AEA is inhibited by co-application with the TRPV1 antagonist capsazepine. AEA also causes maximal vasorelaxation of human pulmonary arteries (Kozlowska *et al.,* 2007). The mechanisms behind AEA-induced relaxation were not probed in the study of Kozlowska *et al.* (2007).

5.2. Aims

In Chapter Three it was suggested that the vasorelaxation produced by AEA in human mesenteric arteries was significantly less than that seen in animals. Therefore, the first aim was to assess the potential role of metabolism in limiting AEA-induced vasorelaxation (Ho and Randall 2007). The second aim was to characterise the AEA-induced vasorelaxation. The third aim was to assess whether AEA causes time-dependent PPARγ-mediated vasorelaxation of human mesenteric arteries (O'Sullivan *et al.,* 2009). Finally, given the findings in chapters three and four, and in light of the often altered levels of AEA in cardiovascular disease or disease risk factors, the fourth aim was to look at AEA-induced vasorelaxation in respect of patient medical condition.

5.3. Methods

5.3.1. Patient consent and arterial preparation

Informed consent was taken from patients (n=32) receiving colorectal re-sections for cancer (n=17) and inflammatory bowel disorders (n=11) as described in section 2.2. Four patients consented to the use of their arteries in this research but declined research access to hospital notes. Tissues were dissected, removing small mesenteric arteries that were either used fresh (n=24) or after overnight storage (n=8) as described in section 2.3

5.3.2. Myography Experiments

Arteries were mounted onto a Mulvany-Halpern myograph and subjected to normalisation and the standard start procedure as described in section 2.3 and 2.4. Cannabinoid concentration-response curves were carried out in viable arteries and compared to vehicle

controls from an adjacent arterial segment from the same patient. To reveal potential limiting factors in AEA-induced vasorelaxation or to characterise the mechanisms of action of AEA, a range of pharmacological techniques were used as described in section 2.7, with comparisons made between responses in test and control arteries taken from the same patient.

In some arteries, potential time-dependent effects of AEA were investigated. In studies using rats, PPARγ-mediated vasorelaxation was only observed in conduit arteries (O'Sullivan *et al.*, 2006). Therefore, in these experiments, larger arteries (>1 mm) were mounted onto fixed hooks and a single concentration of 10 μM AEA (5 μM concentration was shown to cause PPARγ-mediated effects in rat aorta (O'Sullivan *et al.*, 2009)) (in the presence or absence of GW 9662 1 μM) or 0.1 % EtOH was added to the myograph chamber. Readings were taken every 15 minutes to assess potential time-dependent vasorelaxation.

5.3.3. *Post-hoc* Analysis

Post-hoc analysis was performed on control AEA responses from patients who granted access to personal details and medical notes (n=28). Arterial AEA responses were analysed depending on the patient's medical history and medications. Particular attention was paid to diseases and medications that have either a direct vascular impact or are vascular disease risk factors.

5.3.4. Statistical Analysis

Mean percentage relaxation is displayed with error bars representing the s.e.m, and n equalling the number of patients. Sigmoidal concentration-responses curves with a standard Hill slope of 1 were fitted to those data using GraphPad Prism. Statistical comparisons between test and control concentration-response curves and time-dependent experiments were made using 2-way ANOVA.

5.4. Results

5.4.1. The Acute Actions of AEA

The characteristics of patients used in this study are given in table 5.1. AEA caused vasorelaxation with an R_{max} of 32 ± 2% and a pEC_{50} of 6.0 ± 0.2, n=32 (Figure 5.1A & C). AEA caused a significant vasorelaxation of baseline tone (Figure 5.1B & D). Incubation with indomethacin or URB597 did not modify AEA concentration-response curves (Figure

5.2A & B). Antagonism of the CB_1 receptor using AM251 (100 nM) significantly inhibited AEA-induced vasorelaxation (Figure 5.3A). Antagonism of the CB_2 receptor using AM630 (100 nM) or desensitization of TRPV1 receptors using capsaicin (10 μM) had any effect on AEA-induced vasorelaxation (Figure 5.3B & C). Antagonism of the putative CB_e receptor using O-1918 (1 μM) inhibited AEA-induced vasorelaxation (Figure 5.4C). Removal of the endothelium and incubation of arteries with L-NAME significantly inhibited AEA-induced vasorelaxation (Figure 5.4A & B).

Table 5.1 Patient characteristics, diagnosis and medications AEA study

Characteristic	Range	Mean ± s.e.m
Ethnicity	28 UK white	
Male	22	
Female	6	
Age	32 - 82	66 ± 2.1
Weight (kg)	49 - 122	80 ± 4
BMI (kg/m^2)	17.6 - 36.7	27.4 ± 1
Vessel size (μm)	346 - 1372	763 ± 46
Bradykinin response (% reduction in pre-imposed tone)	70 - 102	84 ± 1.6
Smoking habits		
Non smokers	22	
0 - 10 CPD	3	
10 - 20 CPD	3	
Drinking habits		
< 10 units p/w	18	
10 - 20 units p/w	8	
> 20 units p/w	2	
Operation		
Right hemicolectomy	7	
Left hemicolectomy	2	
Sigmoid colectomy	7	
Anterior resection	7	
Abdominoperineal resection	1	
Total colectomy	4	
Reason for surgery		
Cancer	17	
Inflammatory bowel disorder	11	
Dukes staging		
Dukes A	9	
Dukes B	4	
Dukes C	3	
Dukes D	1	
Systolic blood pressure (mm/Hg)	110 - 172	142 ± 3
Diastolic blood pressure (mm/Hg)	65 ± 101	83 ± 2
Diabetic	8	
Heart disease	22	
Heart failure	0	
Hypercholesterolaemia	14	
Hypertensive	16	
α-1 adrenoceptor antagonist (total)	1	
Alfuzosin	0	
Terazosin	1	
ACE inhibitors (total)	7	
Lisinopril	5	
Ramipril	2	
AT1 receptor antagonists (total)	2	
Losartan	1	
Irbesartan	1	
Beta Blockers (total)	5	
Metoprolol	1	
Atenolol	3	
Propranolol	1	

Characteristic	Range	Mean ± s.e.m
Calcium channel blocker (total)	2	
Amlodipine	1	
Nifedipine	1	
Lodipine	0	
Digoxin	1	
Diuretics (total)	2	
Furosemide	2	
GTN	3	
Hypoglycaemic medication (total)	5	
Gliclazide	5	
Metformin	4	
Nsaid medication (total)	13	
Aspirin	5	
Ibuprofen	1	
Paracetamol	4	
Co Codamol	3	
Statin (total)	15	
Atorvastatin	4	
Simvastatin	10	
Pravastatin	1	
Thiazolidinedione (total)	1	
Pioglitazone	1	

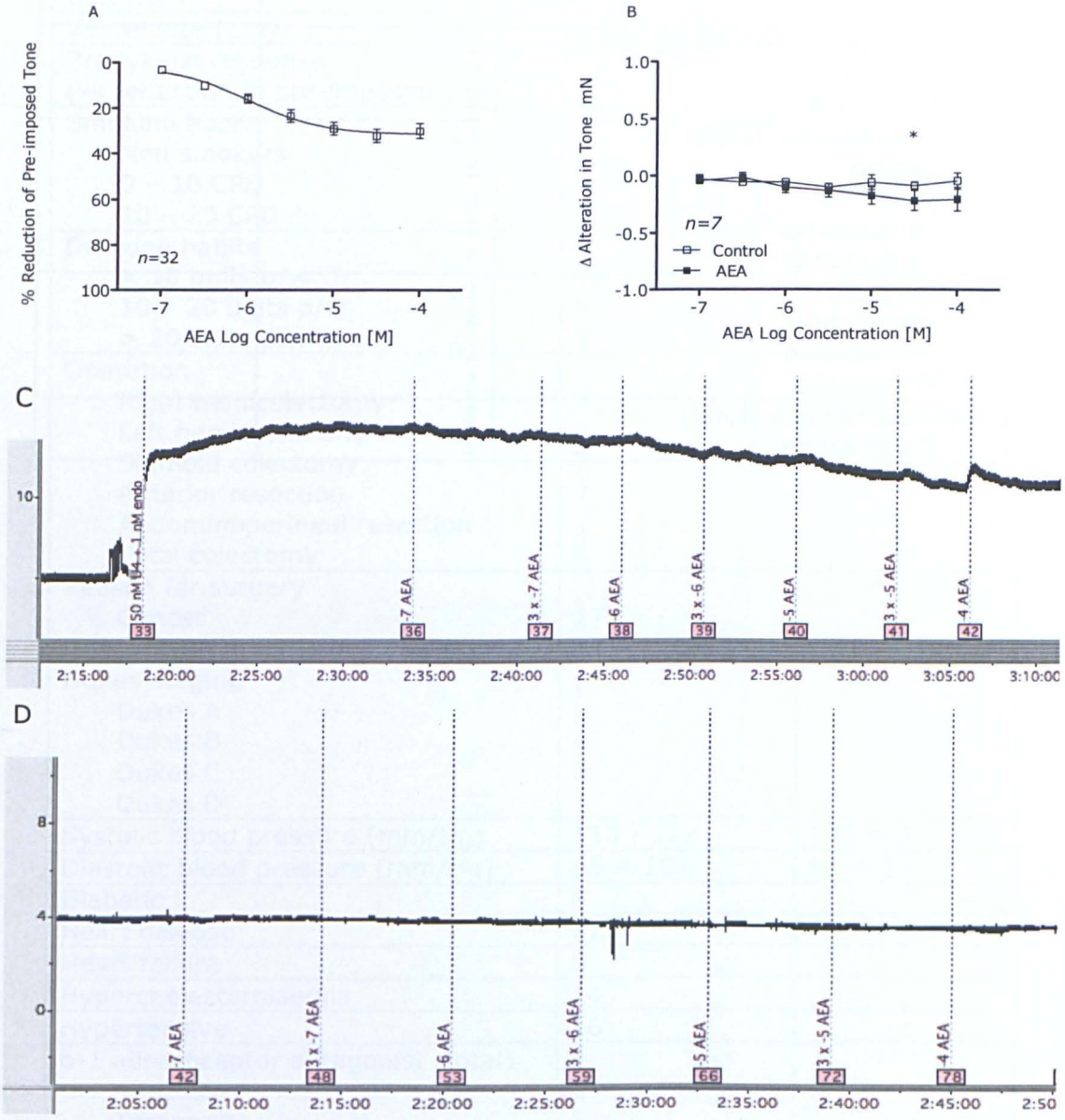

Figure 5.1 AEA responses
AEA-induced vasorelaxation in all patient samples with representative trace (A and C). Effects of AEA on baseline compared to vehicle control and representative trace (B and D). Comparisons were made using 2-way ANOVA.

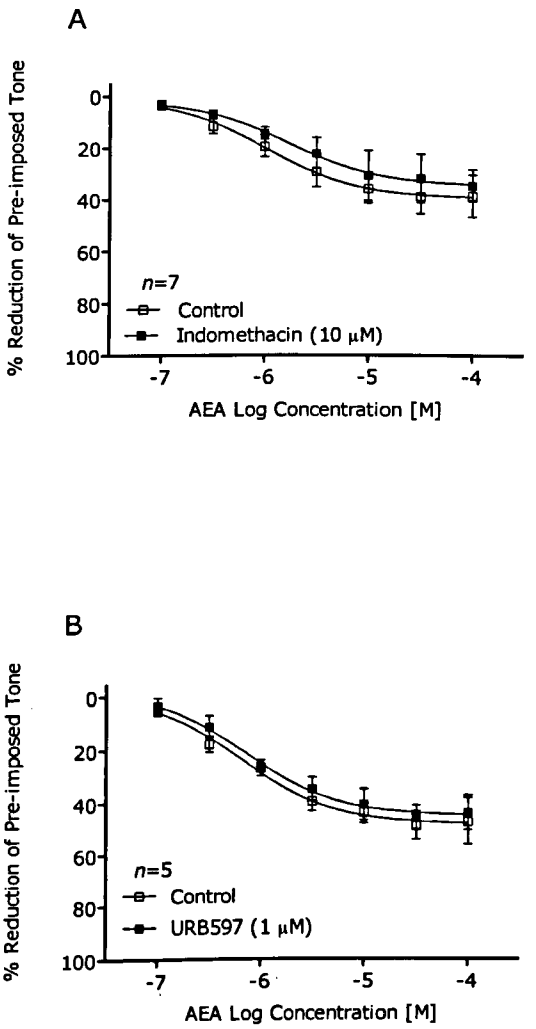

Figure 5.2 Potential role for metabolism in limiting AEA responses AEA-induced vasorelaxation in arteries that have been incubated with the non-selective COX inhibitor indomethacin (A) or the FAAH inhibitor URB597 (B). Comparisons were made using 2-way ANOVA.

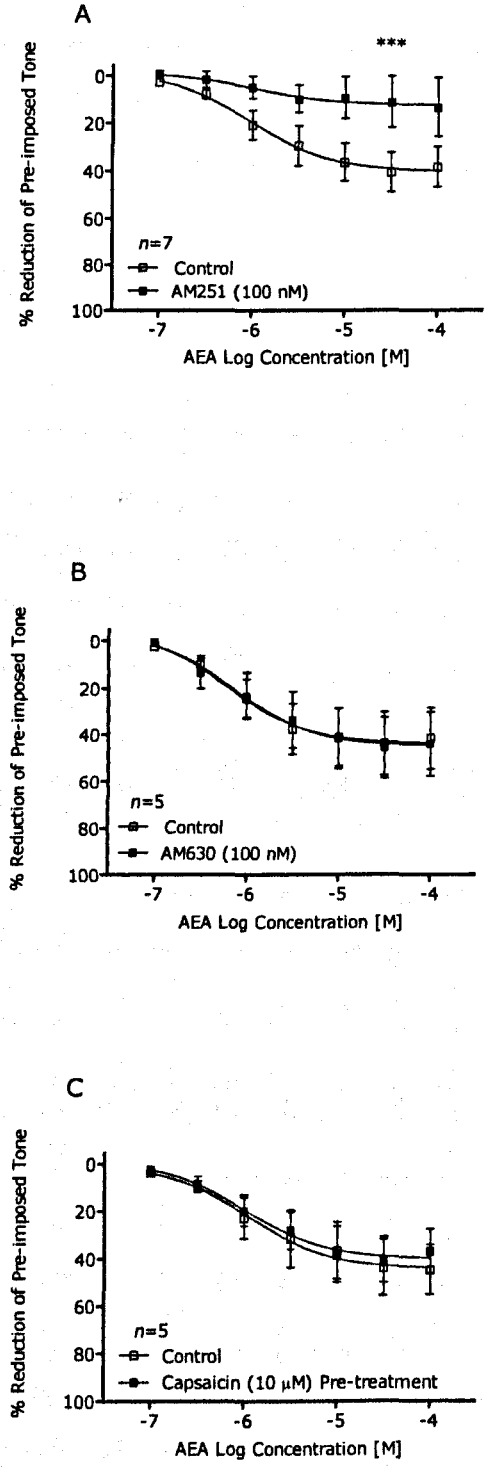

Figure 5.3 Potential receptor involvement in AEA responses
AEA-induced vasorelaxation in arteries incubated with the CB_1 antagonist AM251 (A), the CB_2 antagonist AM630 (B) and in arteries pre-treated with the TRPV1 agonist capsaicin (C). Comparisons made using 2-way ANOVA, ***P<0.001.

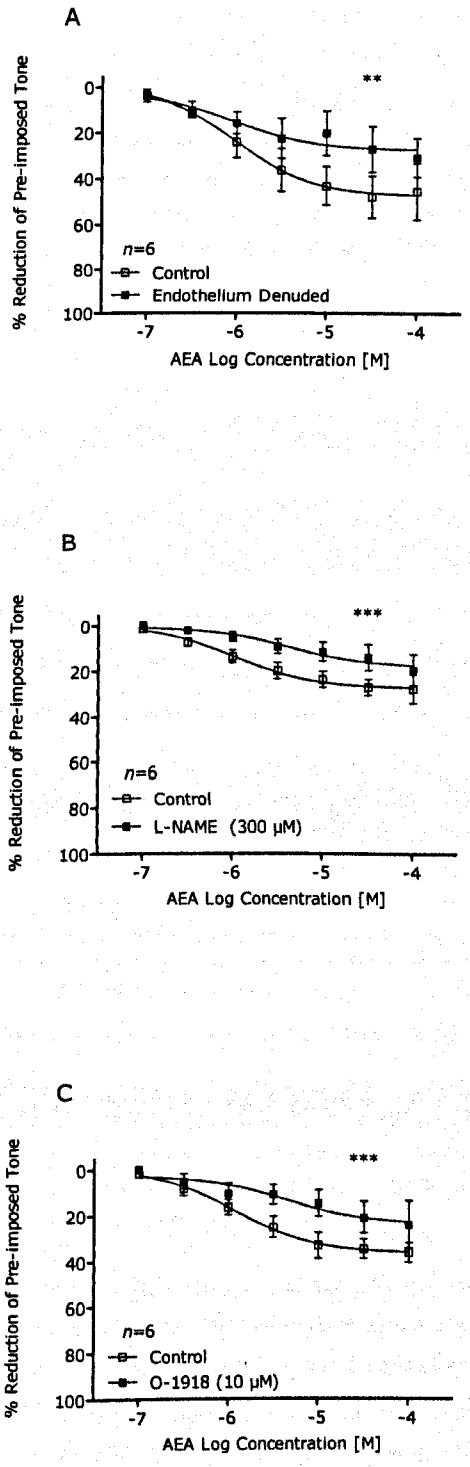

Figure 5.4 Potential endothelium involvement in AEA responses AEA-induced vasorelaxation after endothelial denudation (A), in arteries that have been incubated with the nitric oxide synthase inhibitor L-NAME (B) or in arteries incubated with an antagonist of the proposed putative endothelial cannabinoid receptor (C). Comparisons were made using 2-way ANOVA, ** $P<0.01$ and *** $P<0.001$.

5.4.2. Time-dependent vasorelaxation to AEA

In arteries (>1 mm), a single concentration of 10 μM AEA caused significant vasorelaxation compared to vehicle control (Figure 5.5A). An initial vasorelaxation of 23 ± 3 % relaxation was observed at 15 minutes, this developed to 70 ± 10 % relaxation at 120 minutes (Figure 5.5A). In the presence of the PPARγ antagonist GW9662, vasorelaxation was not inhibited at any time point compared to AEA alone (Figure 5.5B).

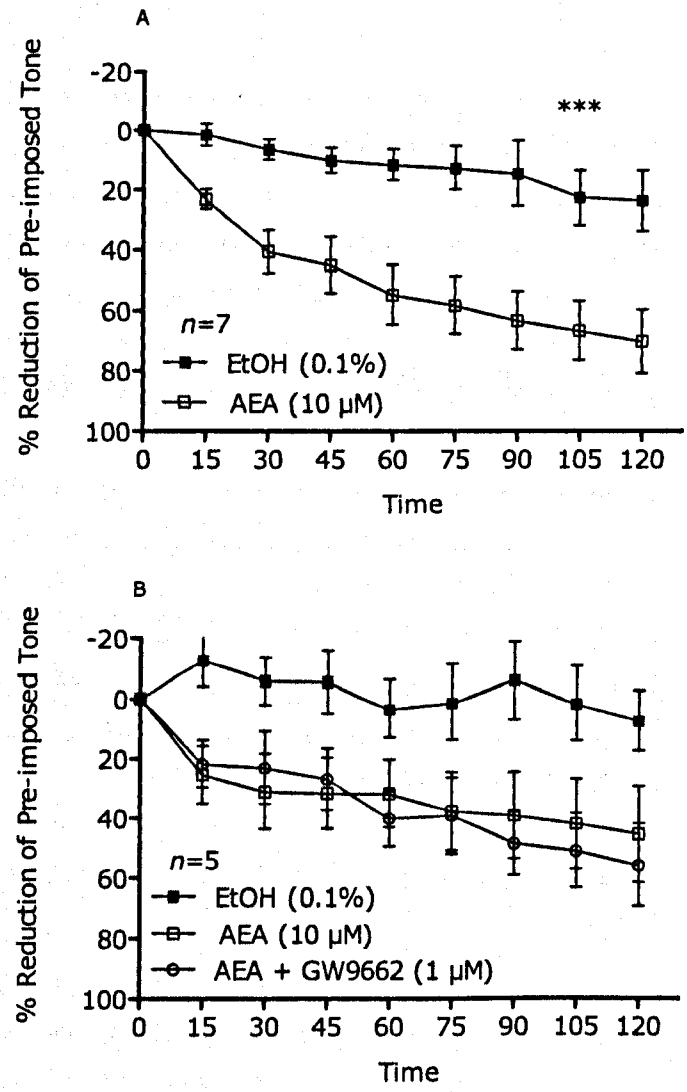

Figure 5.5 Potential time-dependent effects of AEA
Time-dependent vasorelaxation to a single concentration of AEA compared to ethanol vehicle control (A) and in the presence of GW9662 (B). Comparisons were made using 2-way ANOVA, *** $P<0.001$.

5.4.3. *Post-hoc* analysis of patient medical notes

Due to the variability of control responses to AEA among patients (vasorelaxant response range at 100 µmol/L AEA, -4.8 to 83.1% relaxation) *post-hoc* analysis of patient responses was carried out. AEA responses were not gender-related or correlated with bradykinin responses or age (Figure 5.6A, B & D). AEA responses were greater in patients with cancer compared to those with inflammatory bowel disease (Figure 5.6C). AEA responses were significantly reduced in patients diagnosed with heart disease and a BMI >25 kg/m^2 (Figure 5.7A & E). AEA responses were not reduced in those with hypertension, diabetes and hypercholesterolaemia or in smokers (Figure 5.8B, C, D & F). AEA responses were reduced in those taking NSAIDs and beta-blockers (Figure 5.8A & D) but not those taking ACE inhibitors, statins or hypoglycaemic medications (Figure 5.8B, C & E).

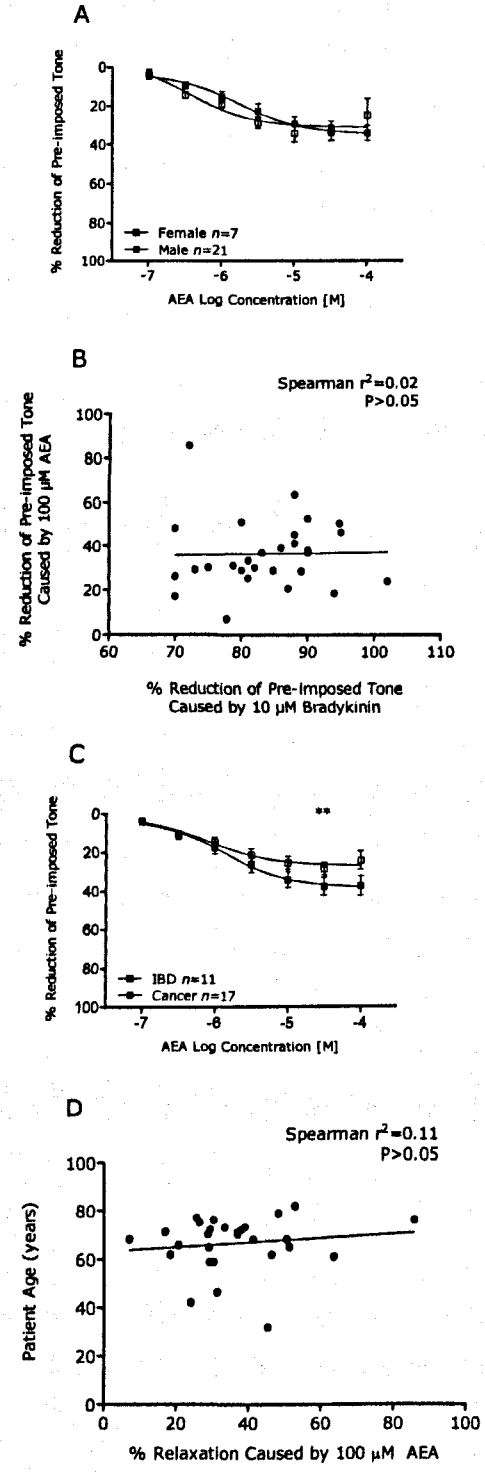

Figure 5.6 Patient characteristics and AEA responses
AEA-induced vasorelaxation in females compared to males (A). AEA R_{max} responses correlated with patient age (B). AEA-induced vasorelaxation in patients with cancer compared to patients without cancer (C). AEA R_{max} responses correlated with bradykinin response from the same artery (D). Comparisons made using 2-way ANOVA and correlations made using Spearman correlation coefficient. **P<0.01.

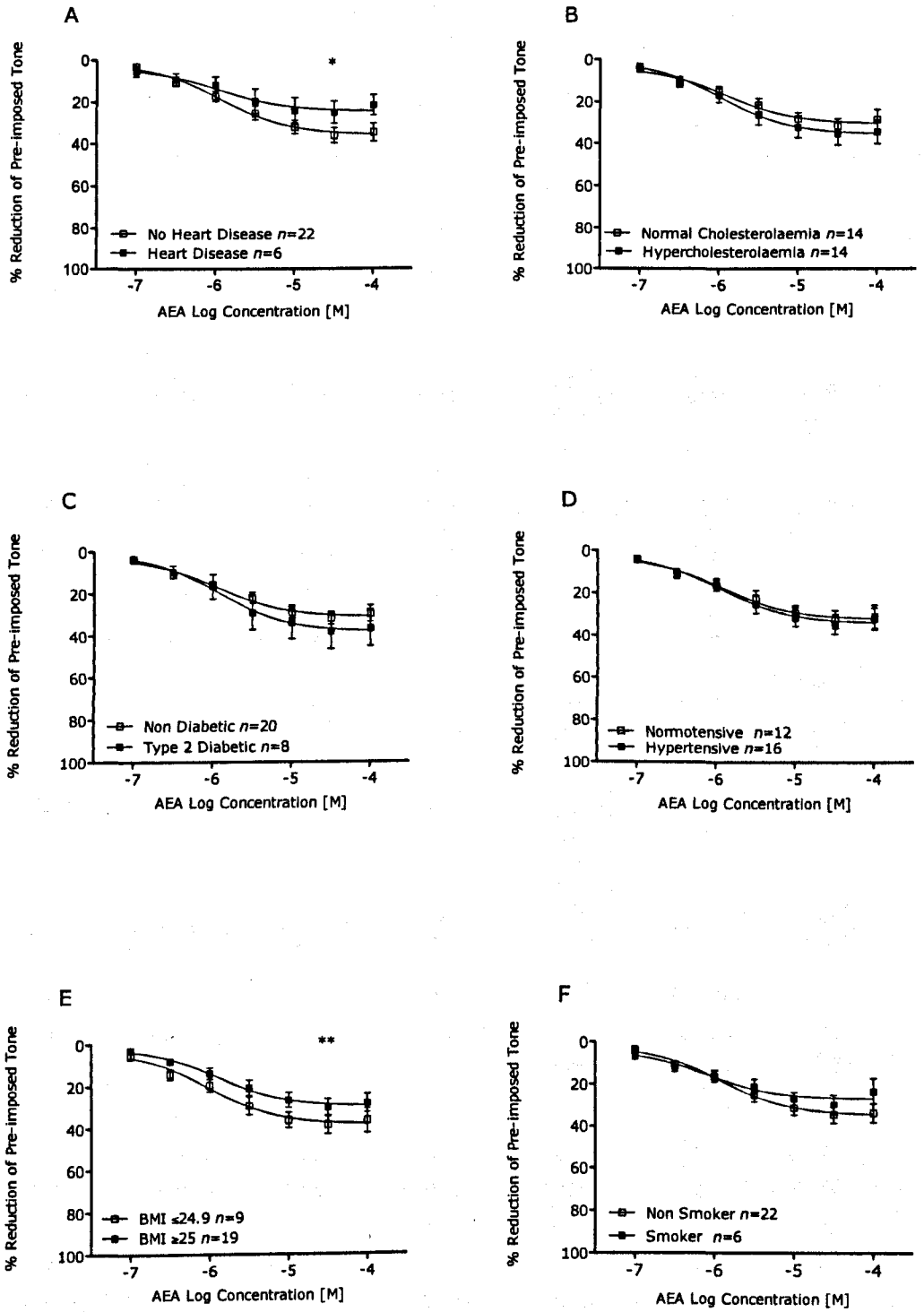

Figure 5.7 Cardiovascular disease/disease risk factors and AEA responses

AEA concentration-response curves in patients with heart disease (A), hypercholesterolaemia (B), type 2 diabetes (C), hypertension (D), elevated BMI (E) and in patients who smoke (F). Comparisons were made between patients with a given characteristic and those without using 2-way ANOVA. * $P<0.05$ and ** $P<0.01$.

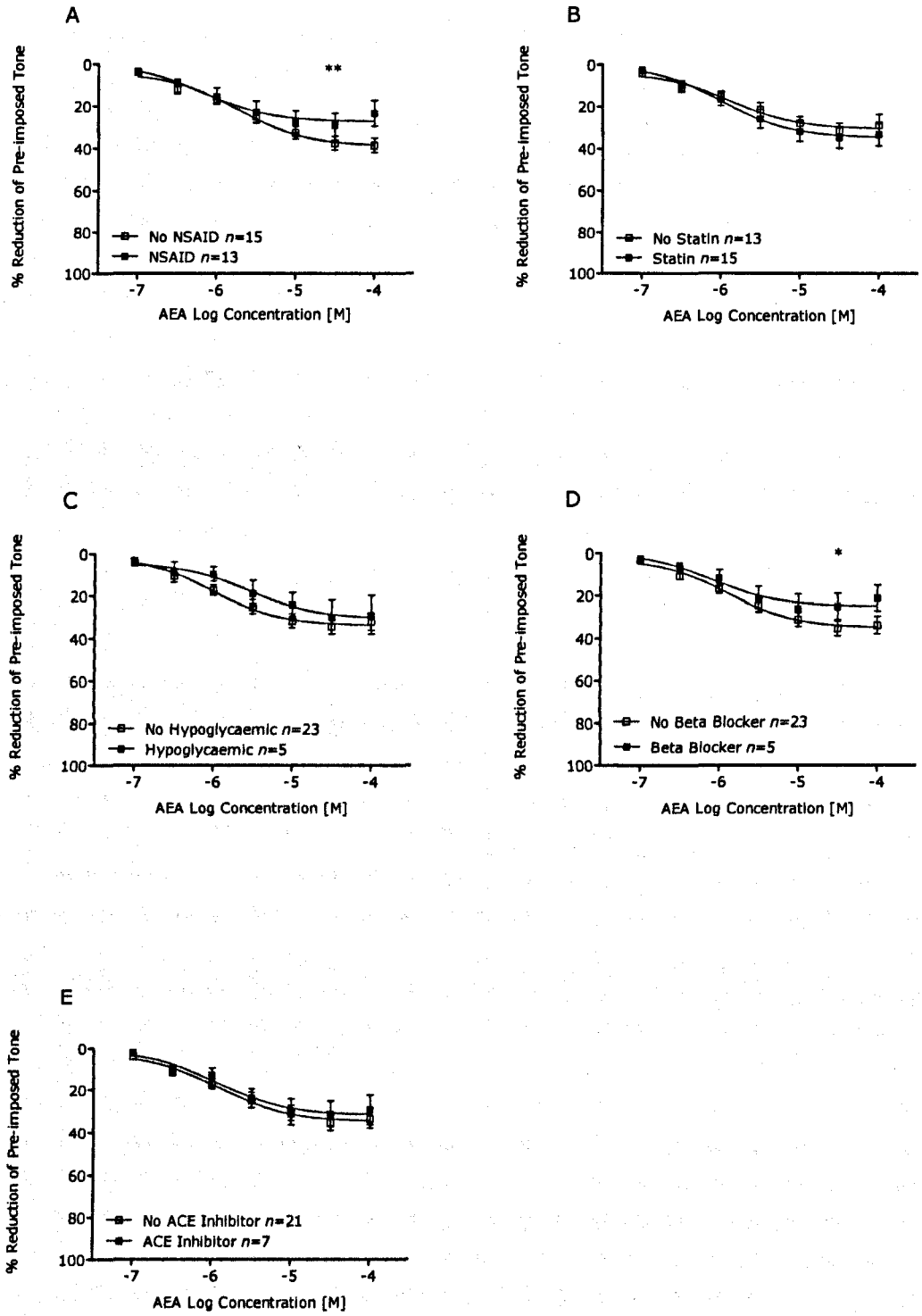

Figure 5.8 Patient medication and AEA responses
AEA concentration-response curves in patients tasking NSAIDs (A), statins (B), hypoglycaemic (C), beta-blocking (D) and ACE inhibiting medication. Comparisons were made between patients taking a given medication and those that were not using 2-way ANOVA. * $P<0.05$ and ** $P<0.01$.

5.5. Discussion

Chapter three showed that although AEA caused vasorelaxation of human mesenteric arteries, the efficacy was reduced when compared to rat mesenteric arteries. A previous study in rat mesenteric arteries reported the vasorelaxant response to AEA could be enhanced by inhibition of AEA metabolism (Ho and Randall 2007). Therefore, the aim of this study was to explore the effects of AEA in human mesenteric arteries. AEA-induced vasorelaxation was not limited by FAAH or COX metabolism. However, AEA-induced vasorelaxation is dependent on CB_1 and CB_e receptor activation, the endothelium and NO. AEA produces vasorelaxation over time, however this is not mediated by the PPARγ receptor. *Post-hoc* analysis revealed that AEA responses were reduced in those with inflammatory bowel disease, ischaemic heart disease, elevated BMI and in patients taking NSAIDs and beta-blockers.

In animal studies, AEA is a potent efficacious vasorelaxant whose effects often depend on the species or the arterial bed studied (Randall *et al.,* 2004). In the present study AEA-induced vasorelaxation in the human mesenteric artery has reduced efficacy compared with rat third order mesenteric arteries (Randall *et al.,* 1997; White and Hiley 1997; O'Sullivan *et al.,* 2004). In other human studies, AEA has been shown to be ineffective in human myometrial arteries (Kenny *et al.,* 2002). However, AEA causes an increase in blood flow in the human forearm when applied topically (Movahed *et al.,* 2005) and *in vitro,* with lower potency than observed in this study, AEA causes maximal vasorelaxation of human pulmonary arteries (Kozlowska *et al.,* 2007). Combined with the previous work, the present study might suggest that the importance of AEA as a regulator of vascular tone depends on the vascular bed, and that its overall systemic effect may be minor. However, it should be noted that the patients that were used in this study were elderly and often had several medical conditions. In young healthy volunteers Movahed *et al.* (2005) observed that arterial infusion of AEA did not cause any alteration in systemic blood pressure or heart rate. This suggests that under otherwise healthy conditions the systemic cardiovascular effect of AEA may be limited. However, local increase in forearm microcirculation may not translate into systemic changes.

Other researchers have shown that the vasorelaxant effects of AEA could be enhanced by inhibition of its metabolism (Ho and Randall 2007). Therefore, we investigated the possibility that the reduced vasorelaxation observed might be a result of AEA being metabolised before reaching potential vasorelaxant targets. Ho *et al.* (2007) showed that incubation with the irreversible FAAH inhibitor URB597 or the COX-2 selective inhibitor nimesulide potentiated the potency of AEA, but not the maximal effect. In contrast, the present study found that inhibiting AEA metabolism using URB597 and the non-selective COX inhibitor indomethacin had no effect AEA-induced vasorelaxation. This suggests that the decreased efficacy of AEA seen in human mesenteric arteries is not due to AEA metabolism. It also shows that the vasorelaxation seen to AEA in human mesenteric arteries is not due to AEA metabolites.

In rat mesenteric arteries, the vasorelaxant response to AEA is sensitive to CB_1, CB_e antagonism and TRPV1 desensitisation (O'Sullivan *et al.*, 2004). Therefore, experiments were carried out to characterise potential involvement of these receptors in the vasorelaxant effects of AEA in human mesenteric arteries. In these experiments it was revealed that AEA-induced vasorelaxation was dependent on activation of the CB_1 and CB_e receptors, but not CB_2 or TRPV1 receptors. In CB_1 and CB_2 transfected CHO cells, AEA binds to the CB_1 and CB_2 receptors with K_i values of 89 and 371 nM respectively (Showalter *et al.*, 1996) and has previously been shown to cause vasorelaxation through these receptors (Randall *et al.*, 1997; White and Hiley 1997; O'Sullivan *et al.*, 2004). CB_1 receptors are present in human vascular endothelium and smooth muscle cells (Sugiura *et al.*, 1998; Liu *et al.*, 2000) and in chapter 4 CB_1 receptors were shown to have vasorelaxant abilities. Therefore, considering AEA has a high affinity for the CB_1 receptor it is not suprising AEA causes vasorelaxation in such a way. AEA-induced vasorelaxation was not inhibited by antagonism of the CB_2 receptor. This, taken with the results of chapter three that HU-308 did not cause vasorelaxation, suggests that in human mesenteric arteries CB_2 receptors are not involved in vasorelaxation. Similarly, in rat mesenteric arteries the effects of AEA are not inhibited by CB_2 antagonism (O'Sullivan *et al.*, 2004).

AEA is an agonist at the TRPV1 receptor with a pEC_{50} value in the low to mid micromolar range (Ross *et al.*, 2001), TRPV1 receptors

are expressed in human smooth muscle and endothelial cells (Fantozzi *et al.,* 2003; Wang *et al.,* 2008). In the rat mesenteric artery, AEA-induced vasorelaxation is inhibited by desensitisation of TRPV1 (O'Sullivan *et al.,* 2004). However, in this study AEA-induced vasorelaxation was not attributable to TRPV1 activation. In chapter four, CBD-induced vasorelaxation was inhibited by TRPV1 desensitisation, suggesting that TRPV1 channels are present and able to induce vasorelaxation of human mesenteric arteries. It is reported that AEA activation of TRPV1 can be altered depending on PKA and PKC activity (De Petrocellis *et al.,* 2001), therefore the lack of TRPV1 activity seen in this study may be associated with altered receptor function. It also been shown that activation of the TRPV1 receptor by AEA requires facilitated transport across the cell membrane (De Petrocellis *et al.,* 2001) and pharmacological inhibition of the proposed membrane transport has been shown to reduce AEA potency in the rat mesenteric artery (Andersson *et al.,* 2002). It is of note that when TRPV1 receptors are desensitised in rat mesenteric arteries there is a similar remaining vasorelaxation to that observed in human mesenteric arteries (O'Sullivan *et al.,* 2004). Therefore, it is possible that the reduced vasorelaxation to AEA in human mesenteric arteries is caused by failure of AEA to activate TRPV1 channels in these arterial segments.

Removal of the endothelium has been shown to inhibit AEA-induced vasorelaxation in rat mesenteric arteries (O'Sullivan *et al.,* 2004; Poblete *et al.,* 2005; Wheal and Randall 2009), rat pulmonary arteries (Baranowska-Kuczko *et al.,* 2012), rabbit aortae (Mukhopadhyay *et al.,* 2002) and bovine ophthalmic arteries (Romano and Lograno 2006). This study similarly shows that removal of the endothelium and inhibition of NO inhibits AEA responses in human mesenteric arteries. Further support for the ability of AEA to cause NO release has been shown in rat first order mesenteric arteries (Wheal and Randall 2009), rat renal arteries (Deutsch *et al.,* 1997), rat pulmonary arteries (Baranowska-Kuczko *et al.,* 2012) and bovine ophthalmic arteries (Romano and Lograno 2006). A further endothelial site known to mediate AEA induced vasorelaxation is the CB_e receptor. AEA causes vasorelaxation through this receptor site in a variety of vascular beds (Jarai *et al.,* 1999; Mukhopadhyay *et al.,* 2002; Begg *et al.,* 2003; Hoi and Hiley 2006). Furthermore this receptor site has been shown to mediate vasorelaxation to the endocannabinoid virodhamine

in the human pulmonary artery (Kozlowska *et al.*, 2008). In the Kzolowska *et al.* (2008) study, arterial incubation with O-1918 reduced the maximal response to virodhamine by almost half. Whereas in rat mesenteric arteries O-1918 shifts the potency of AEA by ten-fold without reducing maximal response (O'Sullivan *et al.*, 2004). In the present study O-1918 caused a 3.3 fold rightward shift in the concentration response curve to AEA, without effecting maxiamal responses. This finding suggests a role for the CB_e receptor in human mesenteric arteries. Furthermore, this finding suggests that activation of the CB_e receptor by AEA in human mesenteric arteries is similar to that observed in rat mesenteric artereis, but not human pulmonary arteries. Both CB_1 and CB_e receptor activation has previously been speculated to produce nitric oxide (Deutsch *et al.*, 1997; Mukhopadhyay *et al.*, 2002). Therefore, given the findings of this study it is likely that, in part, AEA activities either CB_1 or CB_e receptors, which through unknown mechanisms result in endothelial nitric oxide release and subsequent vasorelaxation.

This study reports that a 2-hour incubation with AEA causes vasorelaxation that gradually increases and is significantly different to vehicle and previous time points. However, this effect is not inhibited by PPARγ antagonism. In rat aortae, AEA and NADA cause time-dependent vasorelaxation that can be inhibited by PPARγ antagonism (O'Sullivan *et al.*, 2009). Despite this study showing the time-dependent effect of AEA is not inhibited by PPARγ antagonism, it does not rule out the potential effects of AEA at the PPARγ receptor in human mesenteric arteries. The lack of PPARγ mediated response could be on account of the arteries used. It has previously been shown that in G3 resistance arteries THC is unable to cause PPARγ-mediated vasorelaxation (O'Sullivan *et al.*, 2006), suggesting that PPARγ mediated vascular responses may be restricted to particular arterial beds.

The effects of AEA were not dependent on gender or correlated with age or bradykinin response, a finding that is surprising given that endothelial denudation inhibits vasorelaxation to AEA.

This study shows that the vasorelaxation to AEA is increased in patients with cancer compared to those with inflammatory bowel disease. In colorectal cancer tumour tissues and surrounding mucosal tissue it is reported that the levels of endocannabinoids are higher than

in non-cancer tissues (Ligresti *et al.,* 2003), and it has previously been shown that co-administration of AEA with other endocannabinoids facilitates AEA-induced hypotension through CB_1-sensitive mechanisms (Garcia Mdel *et al.,* 2009). Therefore, even moderately increased endocannabinoid production may act by having a priming effect at the CB_1 receptor and potentiate the effects of CB_1 activation. However, in this case it would be reasonable that CBD responses were enhanced in cancer patients, however the results in chapter four show they were not. An alternative explanation may be that AEA responses are reduced in inflammatory bowel disease. Little is known about the endocannabinoid system in these conditions however, it is known that AEA levels are often altered in mucosal and submucosal tissues (Alhouayek and Muccioli 2012).

This study has also shown that AEA-induced vasorelaxation is similar in patients with hypercholesterolaemia, diabetes and hypertension when compared to patients without the aforementioned characteristics. Interestingly, smoking had no effect on AEA-induced vasorelaxation. Increased cholesterol (Vanhoutte 1991), diabetes (De Vriese *et al.,* 2000), hypertension (Vanhoutte 1996) and smoking (Vanhoutte *et al.,* 2009) are all associated with endothelial dysfunction, reduced nitric oxide production and increased production of vasoconstrictor prostaglandins. However, AEA responses were unaltered in these conditions.

The effects of AEA were reduced in patients with ischaemic heart disease and in patients with increased BMI. In rat mesenteric arteries, cardiac ischaemia and reperfusion were associated with decreased endothelium-dependent vasorelaxation, increased contractile responses and oxidative stress (Zhao *et al.,* 2012). There is also a strong immune response associated with ischaemic heart attacks, whereby the immune system over-compensates the repair of damaged arteries reducing arterial compliance (Timmers *et al.,* 2012). Therefore, in ischaemic heart disease, increased oxidative damage may lead to arterial re-modelling and subsequent reduced vasorelaxation. Interestingly, the effects of CBD were not reduced in patients with IHD, however the effects of both endocannabinoids (AEA and 2-AG) used in this study were reduced in patients with IHD. A further point of interest is that endocannabinoids are increased and associated with coronary circulation dysfunction in ischaemic heart disease (Quercioli *et al.,*

2011). Although the potential reason for this is untested, it is speculated that increased endocannabinoids may chronically stimulate CB_1 leading to increased atherosclerosis and reduced endothelial function. It is also to be noted that in this study all patients with ischaemic heart disease had other vascular co-morbidities including hypertension, diabetes and hypercholesterolaemia. Therefore, a potential explanation for the reduced responses to AEA might be through the extent of vascular damage caused by the summation of several disease states (although none alone affected AEA responses).

This study also observed decreased vasorelaxation to AEA in patients with a BMI >25kg/m^2. Interestingly a recent study has shown that AEA levels are increased in patients that are obese, and that this increase in AEA is directly proportional to endothelial dysfunction in coronary arteries (Quercioli *et al.*, 2011) as discussed above. Given the finding of Quercioli *et al.* (2011), it should not be ruled out that the increase in endocannabinoid levels might have detrimental effects on the cardiovascular system.

This study has shown that AEA-induced vasorelaxation is reduced in patients taking beta-blockers and in patients taking NSAID medication. Given that the effects of AEA are not dependent on COX metabolism, the reduction observed may be caused by the disease states that these medications are used to treat. However, various NSAIDs have been shown to affect the endocannabinoid system (Paunescu *et al.*, 2011), with specific interactions observed between nimesulide, parecoxib and valdecoxib at the CB_1 receptor (Staniaszek *et al.*, 2010; Schroder *et al.*, 2011). Therefore, through as yet unknown mechanisms, the CB_1 mediated responses seen in this study might be altered in the presence of COX inhibitors. Another explanation could be that NSAIDs are reported to inhibit PGI_2 production and therefore contribute to an overall decline in vascular function (McGettigan and Henry 2011).

In conclusion, this study has shown that in human mesenteric arteries, AEA induces vasorelaxation that is less efficacious than seen in animal models. Furthermore, the efficacy of AEA is considerably less than 2-AG (Chapter 3). AEA-induced vasorelaxation occurs through previously shown pathways, but does not activate TRPV1 channels. The reduced effects of AEA may be a result of lack of TRPV1 activity. AEA-induced vasorelaxation is reduced in patients with IHD, increased

BMI and those taking beta-blocker or NSAID medication. Taken together, this study suggests that AEA may act as regulator of vascular tone through known receptors and a potentially unknown uncharacterised receptor in human mesenteric arteries. Furthermore, the effects of AEA may be reduced in patients with cardiovascular disease or taking medication for cardiovascular disease.

6. GENERAL DISCUSSION

Cannabinoids have been shown to cause vasorelaxation of a variety of animal vascular beds in a range of species (reviewed in Chapter 1). The extent of vasorelaxation and the mechanisms utilised often depend on the species, vascular bed or cannabinoid ligand studied. However, the effects of cannabinoids in the vasculature of humans have not been fully investigated. To date there has been only four papers published that have investigated the vascular effects of cannabinoids in human arteries. These papers have looked at a limited range of cannabinoid ligands in vascular beds that have not been commonly tested in animals. Therefore, to fully understand the role or potential therapeutic role of cannabinoids or the endocannabinoid system in the modulation of vascular tone it is important more human work is carried out. The aim of this thesis was to assess the vascular effects of a range of cannabinoid ligands in human mesenteric arteries. Given that the mesenteric vascular bed is the most widely tested in previous cannabinoid studies, human work in this vascular bed enables comparisons to be made more easily between human and animal studies. Once initial screening studies were carried out, a second aim of this thesis was to investigate the mechanisms of action of the most interesting cannabinoid ligands. A third aim of this thesis was to investigate the potential ability of cannabinoids to cause time-dependent PPARγ mediated vasorelaxation of human mesenteric arteries. A final aim of this thesis was to investigate the often seen variability of cannabinoid responses between patients.

6.1. Interpretation of Findings

6.1.1. Cannabinoid Screening Experiments

Initial screening experiments probed six cannabinoid ligands in their potential vascular effects. The cannabinoid ligands chosen were CP55,940, HU308, AEA, 2-AG, Δ^9-THC and CBD. All these ligands with the exceptions of CBD and HU308 have been shown to cause well-characterised vasorelaxation in animal arteries. Screening experiments revealed that the most efficacious cannabinoid tested was the highly potent, highly efficacious CB_1/CB_2 receptor agonist CP55,940. However the CB_2 specific agonist, HU308, was unable to cause vasorelaxation. This finding may suggest that in human arteries

there is a role for the CB_1 receptor but not the CB_2 receptor in causing vasorelaxation. It is also suggestive that any such role for the CB_1 receptor may be modest, as CP55,940 caused ≈50% reduction in pre-imposed tone. Although this study did not probe the mechanisms of action of CP55,940 further support to the speculation for a modest role of the CB_1 receptor can be found in Chapter 5 of this thesis whereby the effects of AEA, a partial agonist at the CB_1 receptor, were in part inhibited by antagonism of the CB_1 receptor. However, confirmation of this theory would be found in further experiments carrying out CP55,940 concentration response curves in the presence of a CB_1 antagonist. Other work could then use other cannabinoid ligands with high CB_1 efficacy such as HU210 to see if a similar level of vasorelaxation was observed to that of CP55,940.

Screening experiments also revealed that 2-AG was the most efficacious endocannabinoid tested. This finding, in human mesenteric arteries, contrasts findings in rat mesenteric arteries where both AEA and 2-AG cause maximal vasorelaxation (Ho and Randall 2007). Therefore the finding that 2-AG causes greater vasorelaxation than that of AEA may be a finding that is specific to the human mesenteric vascular bed, and may suggest that in this vascular bed 2-AG has the ability to regulate vascular tone more than that of AEA. Indeed, in human plasma samples from healthy patients 2-AG concentrations are found to be higher than that of AEA (Quercioli *et al.*, 2011), suggesting that 2-AG is more readily produced than AEA. However the previous study also reported that increased endocannabinoid levels seen in obesity were associated with decreased coronary blood flow (Quercioli *et al.*, 2011). Therefore, to fully understand the role endocannabinoids might play in vascular tone, further studies should address a range of vascular beds and should take arteries from a greater number of patients with and without vascular disease or disease risk factors. Such a study should measure patient plasma and vascular tissue endocannabinoid levels along with the effects of each endocannabinoid in a range of vascular tissues. Such a study should also investigate how specific diseases affected the plasma and tissue endocannabinoid levels along with vascular responses to endocannabinoids. This would aid in developing a broader picture of any potential roles for endocannabinoids in the modulation of vascular tone under both healthy and diseased conditions.

Screening experiments into the effects of two phytocannabinoids (CBD and Δ^9-THC) showed that CBD (a cannabinoid previously uncharacterised in any vascular bed) caused significantly greater vasorelaxation than that of Δ^9-THC. In third order mesenteric arteries THC causes half maximal vasorelaxation (O'Sullivan *et al.,* 2005), whilst in rat mesenteric arteries CBD causes maximal vasorelaxation (Offertaler *et al.,* 2003). Therefore the finding that in humans CBD has a greater maximal effect than Δ^9-THC is in keeping with findings in rat mesenteric arteries. However, the maximal effects of each compound in human mesenteric arteries are lower than those observed in the rat mesenteric arteries.

The main finding of the screening study reported in Chapter 3 was that cannabinoids cause vasorelaxation of human mesenteric arteries. However, striking differences were observed in the maximal effects of cannabinoid ligands used in this study. The differences observed might be associated with the patients that these arteries were taken from, as many of the patients used in this study were advanced in age and presented with several diseases that have a direct impact on vascular function. Although vasorelaxation was validated in this study using bradykinin, this particular vasorelaxant has been shown to be more resistant than other vasorelaxants to vascular decline associated with age or disease (Hatake *et al.,* 1990; DeSouza *et al.,* 2002; Van Guilder *et al.,* 2006; MacKenzie *et al.,* 2008). Indeed, it has been shown that bradykinin produces vasorelaxation of over 70% in older adults and adults with cardiovascular risk factors (Angulo *et al.,* 2012). These findings may suggest that the efficacy of cannabinoid-induced vasorelaxation is more susceptible to vascular decline than that of bradykinin. This might explain why it was often seen that cannabinoid responses might be reduced with responses to bradykinin were unaltered. A further explanation for reduced cannabinoid responses could be accredited to the contractile agent used in this study. In the rat mesenteric vasculature methoxamine is often the contractile agent used (Randall *et al.,* 1997; White and Hiley 1998; O'Sullivan *et al.,* 2004), whilst the present study used U46619. At mid micromolar concentrations (often used in rat mesenteric studies) methoxamine has relatively few off target effects with, to the best of my knowledge, only one paper showing off target actions of methoxamine and that was inhibition of K_{ATP} channels (Haruna *et al.,* 2002). However, at similar

concentrations to those used in this study, U46619 through activation of the TP receptor, has been shown to inhibit BK_{ca} and Kv channels (Cogolludo *et al.,* 2003; Li *et al.,* 2010). Therefore, the choice of contractile agent may have influenced the magnitude of cannabinoid-induced vasorelaxation in this study.

In summary, with the exception of the CB_2 specific ligand, all cannabinoids tested caused vasorelaxation of human mesenteric arteries. However, the maximal effect of cannabinoid-induced vasorelaxation is often less than reported in rat mesenteric arteries. The reduced maximal effect of cannabinoids in humans may be attributed to the patient population from which samples were taken, differences in the methodology, or may simply be attributed to species differences. Finally, although cannabinoid responses in human mesenteric arteries were modest this should not rule out targeting the endocannabinoid system as a potential therapeutic target in the modulation of vascular tone in health and disease. Given that, the regulation of vascular tone is often multifactorial, the endocannabinoid system in this should not be overlooked.

6.1.2. Cannabinoid Mechanisms of Action

The first cannabinoid to be probed for mechanisms of action was the endocannabinoid 2-AG (Chapter 3). These experiments revealed that 2-AG induced-vasorelaxation was distinct from the effects of 2-AG in rat mesenteric arteries. The distinction being that 2-AG-induced vasorelaxation, in rat mesenteric arteries, is limited by metabolism (Ho and Randall 2007). However, in humans, similar to bovine coronary arteries (Gauthier *et al.,* 2005), metabolism mediates 2-AG-induced vasorelaxation. Gauthier *et al.* (2005) report that 2-AG-induced vasorelaxation is dependent on MAGL and FAAH hydrolysis of 2-AG to arachidonic acid and then subsequent metabolism by COX. In human mesenteric arteries the MAGL and FAAH inhibitors JZL184 and URB597 had no effect on 2-AG-induced vasorelaxation. This suggests that 2-AG was directly metabolised by COX. Further experiments to find the particular COX isoform involved in 2-AG-induced vasorelaxation revealed that the COX-2 specific inhibitor nimesulide had no effect on vasorelaxation, whereas the COX-1 favourable inhibitor flurbiprofen reduced 2-AG-induced vasorelaxation. This finding is in contention with research showing COX-1 and COX-2 binding affinities for 2-AG (Kozak

et al., 2001). Work suggests that COX-1 lacks 2-AG affinity due to the amino acids present in residue 513 of the side pocket-binding region. In this residue COX-1 has a histidine amino acid whereas COX-2 has an arginine. Mutations in Arg-513 significantly reduce COX-2 affinity for 2-AG (Kozak *et al.*, 2001). Given that Kozak *et al.* (2001) use human COX-1 and COX-2 in their work, it is likely that there are potential issues in the pharmacology of the COX inhibitors used in Chapter 3. For instance, it has been shown that the main amino acid residue involved in the specific binding of COX-2 inhibitors is that of valine 523, and that some COX-2 inhibitors, depending on chemical structure, do not bind to Arg-513 (DeWitt 1999). Further work should look at the effects of a range of COX-2 inhibitors on the effects of 2-AG-induced vasorelaxation. Such studies would be of therapeutic interest in that they might allow 2-AG access to its site of metabolism on COX-2 that may elicit vasorelaxation, yet also reduce the detrimental effects of COX-2 metabolism of arachidonic acid. A further finding of interest was that 2-AG-induced vasorelaxation was enhanced in the presence of AH6809 (an antagonist at both contractile and vasorelaxant prostanoid receptors). This may suggest that 2-AG metabolism produces both vasorelaxant and vasoconstrictor prostanoids, which may also provide a potential therapeutic pathway, in that antagonism of vasoconstrictor prostanoid receptors may enhance vasorelaxation not only to endocannabinoids but also to arachidonic acid metabolites.

In Chapter 5 this thesis explored the effects of metabolism on AEA-induced vasorelaxation. Previous studies in the rat mesenteric artery showed that the effects of both 2-AG and AEA were limited by their metabolism (Ho and Randall 2007). However, Chapter 5 revealed that inhibition of FAAH or COX had no effect on AEA-induced vasorelaxation. Further work to explore the mechanisms of AEA-induced vasorelaxation revealed that AEA-induced vasorelaxation was inhibited by CB_1 and CB_e receptor antagonism. Given that AEA is a partial agonist at the CB_1 receptor this may add strength to the previous argument concerning the speculation to the modest role the CB_1 receptor may play in the regulation of vascular tone. Interestingly, similar to vasorelaxation in the rat kidney (Deutsch *et al.*, 1997) and rabbit aortic ring (Mukhopadhyay *et al.*, 2002), AEA-induced vasorelaxation could be inhibited by antagonism of CB_1/CB_e ($G_{i/o}$-coupled) receptors and was also dependent on nitric oxide.

Suggesting a potential link between these receptors and the production of nitric oxide. Indeed, later work has gone on to show that the metabolically stable synthetic analogue of AEA, methanandamide, stimulates nitric oxide production in endothelial cells through $G_{i/o}$-coupled receptor activation of the phosphatidylinositide 3-kinase protein kinase B pathway (McCollum *et al.*, 2007). Further support for the potential role of the CB_1 receptor signalling to release nitric oxide in the human mesenteric arteries came in Chapter 4 whereby CBD-induced vasorelaxation could be inhibited using AM251, LY320135 and L-NAME. Given that both AEA and CBD implicate a role for the CB_1 receptor and that both ligands are also inhibited by L-NAME this offers a strong suggestion of the presence of a CB_1 receptor in human mesenteric arteries and that this receptor may be coupled to nitric oxide release. Therefore, although the vasorelaxation through this receptor appears modest, this makes the CB_1 receptor a potential therapeutic target of interest given that both these ligands are weak or partial agonists at this receptor. Future studies should investigate the effects of cannabinoids with increased efficacy at this receptor such as CP55,940 and HU210. However, it should be noted that such a therapeutic strategy might have complicated risks. The CB_1 receptor is expressed widely throughout the cardiovascular system, therefore administration of a highly potent, highly efficacious agonist at such a receptor may have detrimental effects. For example, CB_1 antagonism in the cardiovascular system has been shown to reduce myocardial apoptosis (Mukhopadhyay *et al.*, 2007), reduce atherosclerotic lesions (Sugamura *et al.*, 2010) and reduce cell death of human coronary artery endothelial cells (Rajesh *et al.*, 2010). Therefore if such a therapeutic tool were going to be used, careful consideration would need to be paid to several factors including, but not limiting, to the dose, the route of administration and the circumstances leading to administration.

Studies probing the effects of AEA also highlighted the involvement of the putative CB_e receptor. The involvement of this receptor has previously been implicated in other human arteries. In the human pulmonary artery virodhamine and Abn-CBD both cause maximal vasorelaxation that is reduced in the presence of the CB_e antagonist O-1918 (Kozlowska *et al.*, 2007; Kozlowska *et al.*, 2008). However, there is much still unknown about this receptor and its

identification and full characterisation would need to be carried out before considering it therapeutically. The most recent speculation as to the identity of the CB_e receptor comes from the Bradshaw laboratory, whereby it has been shown that the novel GPR18 receptor shares a similar pharmacological profile as the CB_e receptor (McHugh *et al.,* 2012). The isolation and complete characterisation of the CB_e receptor may offer a potentially exciting and therapeutic target in the vasculature given its known vasorelaxant effects with, as yet, no known detrimental effects.

A finding of particular interest was the finding that AEA-induced vasorelaxation was unaffected by desensitisation of the TRPV1 receptor using capsaicin. Confirmation of a role for this receptor was shown in Chapter 4 whereby CBD-induced vasorelaxation was inhibited by 1 hour pre-treatment with capsaicin. Therefore, the lack of role for TRPV1 in AEA-induced vasorelaxation appears to be specific to AEA, and could account for the reduced efficacy seen to AEA in human mesenteric arteries. O'Sullivan *et al.* (2004) reported that, in rat third order mesenteric arteries, desensitisation of the TRPV1 receptor reduced the maximal effect of AEA from 100% reduction in pre-imposed tone to 50% reduction in pre-imposed tone. The lack of a TRPV1-mediated role in AEA-induced vasorelaxation might be specific to humans. Indeed, AEA activation of the TRPV1 receptor is reported to be dependent on the presence of an intracellular transporter (De Petrocellis *et al.,* 2001), the phosphorylation state of the TRPV1 receptor (De Petrocellis *et al.,* 2001) and the temperature at which the experiment is carried out (Sprague *et al.,* 2001). Further studies could specifically probe the effects of AEA at the TRPV1 receptor by carrying out concentration response curves to capsaicin to firstly confirm the presence of the TRPV1 receptor. Then investigations into AEA-TRPV1 interactions could look at the effects of temperature, receptor phosphorylation state and voltage state. This could be done using myography and altering parameters such as temperature settings and buffer solutions. Investigations into the role of an intracellular transport protein in the transportation of AEA is an issue of contention, however it has been suggested recently that fatty acid binding proteins and heat shock proteins may act as such transporters and allow AEA uptake (reviewed in Fowler 2012). Experiments to probe the effects of fatty acid binding proteins and heat shock proteins in humans would be difficult.

However, human mesenteric arteries could be assessed for content of fatty acid binding proteins (FABP5 and FABP7) and heat shock protein (Hsp70), all of which have been shown to increase AEA uptake in cell culture (reviewed in Fowler 2012). Such studies may have revealed reduced levels of these proteins in this tissue and therefore may explain the lack of TRPV1 involvement in AEA-induced vasorelaxation. Pharmacologically targeting TRPV1 sensitivity to AEA activation may prove a useful therapeutic as it would enhance the effects of AEA, whilst avoiding any potential detrimental effects of increased levels of AEA.

A third aim of this study was to assess the potential for cannabinoids to cause time-dependent PPARγ-induced vasorelaxation. This novel mechanism of cannabinoid-induced vasorelaxation was shown using Δ^9-THC in the rat aorta (O'Sullivan *et al.,* 2005), since this paper it has been shown that CBD, AEA and NADA all cause vasorelaxation through PPARγ activation (O'Sullivan *et al.,* 2009; O'Sullivan *et al.,* 2009). Although these effects are not just limited to the aorta they have been shown to be present in conduit arteries as opposed to resistance arteries (O'Sullivan *et al.,* 2006). Therefore experiments were undertaken in the largest human arteries available to see if these effects were present in human mesenteric arteries. These experiments revealed that although CBD and AEA did cause vasorelaxation that significantly increased over a two-hour period, it could not be antagonised using an antagonist of the PPARγ receptor. Whilst there is the possibility that this time-dependent vasorelaxation may have occurred via a different PPAR receptor isoform, such as PPARα (Romano and Lograno 2012), time-dependent vasorelaxation may have occurred through an already identified pathway. For example, CB_1 receptor stimulation with highly potent, highly efficacious ligands leads to rapid internalisation of the receptor often within 20 minutes and at picomolar concentrations (Hsieh *et al.,* 1999; Grimsey *et al.,* 2010). However, when stimulated with less efficacious agonists such as Δ^9-THC and methanandamide have been shown to still have receptors present and signalling on cell surface membranes after half an hour (Hsieh *et al.,* 1999). Suggesting that receptor internalisation is dependent on maximal or near maximal receptor activation. Therefore given that AEA and CBD are both partial/weak

agonists at the CB_1 receptor this may stop the CB_1 receptor internalising and may allow for a continued vasorelaxation.

In summary cannabinoid-induced vasorelaxation occurs through both receptor and metabolism mediated pathways (see Figure 6.1). With AEA sharing similar vasorelaxation pathways to that of the phytocannabinoid CBD, potentially due to it being a more stable compound than 2-AG. This thesis has shown some differences in the mechanisms of cannabinoid-induced vasorelaxation, with these differences potentially explaining the reduced efficacy to AEA. However, the main finding of these studies is that the endocannabinoid system through receptor activation and metabolism is capable of causing vasorelaxation in human mesenteric arteries.

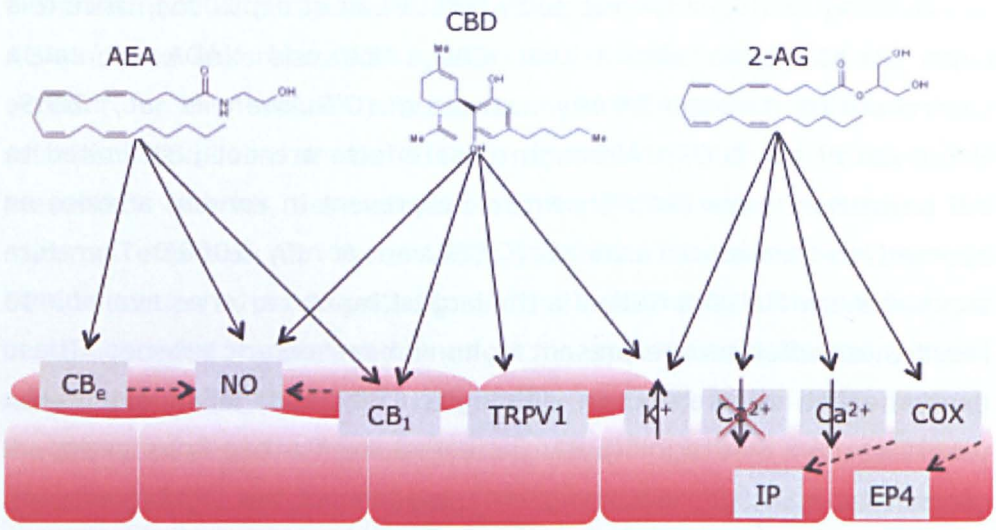

Figure 6.1 Schematic representation of cannabinoid-induced vasorelaxation in human mesenteric arteries

6.1.3. Bearing of Patient Characteristics on Cannabinoid Responses

Cannabinoid responses showed varied degrees of vasorelaxation at the highest cannabinoid concentration used. 2-AG responses ranged from 0-103% reduction in pre-imposed tone (Chapter 3), CBD responses ranged from 22-75% reduction in pre-imposed tone (Chapter 4) and AEA responses ranged from -4.8-83.1% reduction in pre-imposed tone (Chapter 5). In an attempt to understand some of the potential differences in these responses *post-hoc* analysis of patient medical notes were performed. Patient medical notes were assessed for cardiovascular disease or cardiovascular disease risk factors, a

summary of these findings can be seen in table 6.1. The most common reduction in response was in patients taking NSAID medication with 2-AG, AEA and CBD (Chapters 3, 4 and 5) all being reduced. In the studies conducted in this thesis, NSAIDs were the most commonly prescribed drug with a third of patients taking NSAIDs for a range of cardiovascular diseases or cardiovascular disease risk factors. Therefore, the reduced responses observed could be on account of the variety of underlying diseases that the NSAIDs were used to treat. However, NSAIDs have been shown to affect levels of PGI_2 (McGettigan and Henry 2011) and affect binding at the CB_1 receptor (Staniaszek *et al.,* 2010) which are two major pathways of cannabinoid-induced vasorelaxation in human mesenteric arteries. The finding that ischaemic heart disease reduced responses only to the endocannabinoids tested in this thesis, is a finding of interest given that 2-AG and AEA did not share common vasorelaxation pathways (Chapter 3 and 5). However, recent reports have suggested that raised endocannabinoid levels are associated with coronary dysfunction in obesity (Quercioli *et al.,* 2011). Given the findings shown in this thesis it is possible to suggest that the raised endocannabinoids seen by Quercioli *et al.* (2011) might be caused by loss of endocannabinoid sensitivity in the vasculature. However it is also likely that high circulating levels of endocannabinoids desensitise the vascular responses to endocannabinoids. Hypercholesterolaemia and type two diabetes caused reductions in cannabinoid sensitivity to both 2-AG and CBD (Chapters 3 and 4). The possible reasons for these reduced responses may lay in the altered prostaglandin functions and endothelial dysfunction associated with these disease states (Vanhoutte *et al.,* 2009). However, it is of interest that the responses to AEA were not inhibited in such patients (Chapter 5). In hypertensive models the vasorelaxant effects of cannabinoids can be enhanced (see introduction section), however this thesis has shown that responses to 2-AG (Chapter 3) and AEA (Chapter 5) were unaltered, yet responses to CBD (Chapter 4) were reduced in hypertension. Given that the TRPV1 receptor mediated vasorelaxation to CBD but not 2-AG and AEA, and that TRPV1 receptor expression is reduced in models of hypertension (Wang and Wang 2006), this might explain the exclusivity of this finding.

Although cannabinoid responses were often significantly reduced in patients with cardiovascular disease, cardiovascular disease risk factors or in patients taking various medications, some of these findings might have little biological relevance. Indeed, Thompson (2002) argues that statistical significance ($P<0.05$) does not relate to biological significance, as studies are able to manipulate statistical significance to make small effects significant given a large enough sample size. Therefore, to test biological significance, effect sizes must be reported (tables 6.2, 6.3 and 6.4). In assessing the effect size the s.e.m should be viewed more critically, and it is suggested that biological significance would not occur unless the mean of one variable was at least several orders of s.e.m different to the mean of the other variable (Thompson 2002). Given this argument, if the data in tables 6.2, 6.3 and 6.4 were assessed to have biological significance providing that there was greater than two s.e.m between each mean value, there would be no significant differences in cannabinoid potency. However, it would be expected that there was biologically relevant reductions in vasorelaxation to the maximal effects of 2-AG and AEA in patients with heart disease and patients taking NSAID medication (2-AG responses would also be reduced in patients taking hypoglycaemic and statin medication). Furthermore, CBD responses would only be reduced in those with diabetes or on diabetic medication.

In summary the interpretation of the effects of patient disease state on cannabinoid responses should be carried out with caution. There is a clear distinction between statistical significance and biological significance. However, the finding that cannabinoid responses are often extremely variable in humans deserves greater attention. Understanding these variances and the endocannabinoid system in health and disease might provide future therapeutic strategies. Further studies should look at the vascular effects of endocannabinoids in healthy and diseased patients, but they should have strict inclusion and exclusion criteria to limit the potential confounding factors such as multiple diseases or multiple medication. Also, such studies should approach reporting findings with caution and pay considerable attention to statistical tests used in reporting findings.

Table 6.1 The effects of patient disease state on vasorelaxant responses to 2-AG, CBD and AEA in human mesenteric arteries

Patient characteristic	Effect on vasorelaxation		
	2-AG	CBD	AEA
General			
Gender	Enhanced in females	No effect	No effect
Correlation with bradykinin response	Non	Yes	No
IBD patients compared to cancer patients	No effect	No effect	Reduced in IBD
Correlation with patient age	Non	Non	Non
Cardiovascular disease/disease risk factor			
Heart disease	Reduced	No effect	Reduced
Hypercholesterolaemia	Reduced	Reduced	No effect
Type-2 diabetes	Reduced	Reduced	No effect
Hypertensive	No effect	Reduced	No effect
BMI ≥25kg/m^2	No effect	No effect	Reduced
Smoker	No effect	Enhanced	No effect
Medication			
NSAID	Reduced	Reduced	Reduced
Statin	Reduced	Reduced	No effect
Hypoglycaemic	Reduced	Reduced	No effect
Beta blocker	No effect	Reduced	Reduced
ACE inhibitor	No effect	No effect	No effect

Table 6.2 Patient disease states in 2-AG study, alterations in pEC_{50} and R_{max} values. Data given as mean ± s.e.m with *n* equal to the number of patients

Patient characteristic	Effects on 2-AG-induced vasorelaxation				
		Presenting characteristic		Absence of characteristic	
		mean ± s.e.m	*n*	mean ± s.e.m	*n*
Heart disease	pEC_{50}	5.1±0.2	9	5.6±0.1	32
	R_{max}	49.7±3.9		76.3±3.3	
Hypercholesterolaemia	pEC_{50}	5.4±0.2	16	5.6±0.1	25
	R_{max}	65.4±5.0		75.5±3.8	
Type-2 diabetes	pEC_{50}	5.4±0.3	10	5.5±0.1	31
	R_{max}	58±7.1		76.3±3.0	
Hypertensive	pEC_{50}	5.5±0.2	16	5.5±0.1	25
	R_{max}	65±4.1		73±4.1	
BMI ≥25kg/m^2	pEC_{50}	5.4±0.2	13	5.6±0.1	28
	R_{max}	72.5±5.9		70.1±3.7	

Smoker	pEC_{50}	5.2±0.2	11	5.6±0.1	30
	R_{max}	78.0±6.3		68.8±3.5	
NSAID	pEC_{50}	5.2±0.2	14	5.6±0.1	27
	R_{max}	51.1±5.0		81.1±3.1	
Statin	pEC_{50}	5.3±0.2	16	5.6±0.1	25
	R_{max}	57.6±5.0		77.4±3.3	
Hypoglycaemic	pEC_{50}	5.1±0.3	6	5.5±0.1	35
	R_{max}	43.2±5.8		74.8±3.0	
Beta blocker	pEC_{50}	5.3±0.2	8	5.6±0.1	33
	R_{max}	65.1±4.8		69.9±3.5	
ACE inhibitor	pEC_{50}	5.5±0.3	6	5.5±0.1	35
	R_{max}	65.4±6.8		70.7±3.3	

Table 6.3 Patient disease states in CBD study, alterations in pEC_{50} and R_{max} values. Data given as mean ± s.e.m with *n* equal to the number of patients

Patient characteristic	Effects on CBD-induced vasorelaxation				
		Presenting characteristic		Absence of characteristic	
		mean ± s.e.m	*n*	mean ± s.e.m	*n*
Heart disease	pEC_{50}	5.5±0.2	9	5.5±0.1	25
	R_{max}	41.3±4.0		44.0±2.1	
Hypercholesterolaemia	pEC_{50}	5.4±0.2	15	5.6±0.1	19
	R_{max}	40.2±3.2		44.6±2.3	
Type-2 diabetes	pEC_{50}	5.4±0.2	10	5.5±0.1	24
	R_{max}	34.6±2.0		46.8±3.6	
Hypertensive	pEC_{50}	5.4±0.2	16	5.5±0.1	18
	R_{max}	41.8±2.9		44.5±2.4	
BMI ≥25kg/m^2	pEC_{50}	5.5±0.2	24	5.5±0.1	10
	R_{max}	46.1±3.2		42.1±2.3	
Smoker	pEC_{50}	5.5±0.2	6	5.5±0.1	28
	R_{max}	48.7±4.5		42.1±2.1	
NSAID	pEC_{50}	5.4±0.2	14	5.5±0.1	20
	R_{max}	40.9±3.0		44.9±2.4	
Statin	pEC_{50}	5.4±0.2	14	5.6±0.1	20
	R_{max}	39.6±3.1		46.9±2.3	
Hypoglycaemic	pEC_{50}	5.5±0.3	6	5.5±0.1	28
	R_{max}	26.9±3.9		46.9±2.0	

Beta blocker	pEC_{50}	5.4±0.3	6	5.5±0.1	28
	R_{max}	35.3 ±5.0		45.0±2.0	
ACE inhibitor	pEC_{50}	5.5±0.3	7	5.5±0.1	27
	R_{max}	40.0±5.0		44.2±2.0	

Table 6.4 Patient disease states in AEA study, alterations in pEC_{50} and R_{max} values. Data given as mean ± s.e.m with *n* equal to the number of patients

Patient characteristic	Effects on AEA-induced vasorelaxation				
		Presenting characteristic		Absence of characteristic	
		mean ± s.e.m	*n*	mean ± s.e.m	*n*
Heart disease	pEC_{50}	6.0±0.5	6	5.9±0.2	22
	R_{max}	24.3±3.1		35.7±2.0	
Hypercholesterolaemia	pEC_{50}	6.0±0.3	14	5.9±0.2	14
	R_{max}	35.7±2.8		30.8±2.1	
Type-2 diabetes	pEC_{50}	5.9±0.4	8	5.9±0.2	20
	R_{max}	38.1±4.5		31.3±1.7	
Hypertensive	pEC_{50}	5.9±0.3	16	5.9±0.3	12
	R_{max}	34.0±2.6		32.2±2.4	
BMI ≥25kg/m^2	pEC_{50}	5.9±0.2	19	6.0±0.2	9
	R_{max}	30.6±2.3		38.9±2.5	
Smoker	pEC_{50}	6.1±0.4	6	5.9±0.2	22
	R_{max}	27.1±2.4		34.9±2.2	
NSAID	pEC_{50}	6.2±0.4	13	5.7±0.2	15
	R_{max}	27.5±2.8		38.9±2.1	
Statin	pEC_{50}	6.0±0.3	15	5.9±0.3	13
	R_{max}	35.2±2.7		31.1±2.3	
Hypoglycaemic	pEC_{50}	5.5±0.4	5	6.0±0.2	23
	R_{max}	30.8±4.7		33.9±1.9	
Beta blocker	pEC_{50}	6.1±0.4	5	5.9±0.2	23
	R_{max}	25.2 ±3.1		35.1±2.1	
ACE inhibitor	pEC_{50}	5.9±0.3	7	5.9±0.2	21
	R_{max}	31.7±3.1		34.6±2.1	

6.2. Limitations and future work

The focus of this study was to investigate the pharmacological effects of cannabinoids in human mesenteric arteries. To do this, this study utilised mesenteric arteries from patients undergoing colorectal surgery for colorectal cancer or inflammatory bowel disorders. Arteries were then probed pharmacologically whilst mounted on a wire myograph.

One of the major limitations of this study was the single technique used to carry out investigations. Wire myography is often used to characterise vascular function and pharmacological responses, and is an extremely useful tool in that many compounds can be screened concurrently. However, wire myography does not exactly replicate physiology. In studies comparing both wire and pressure myography it has been shown that arteries mounted on a wire myograph often have decreased sensitivity to exogenously applied vasoconstrictors (Buus *et al.*, 1994; Dunn *et al.*, 1994; Falloon *et al.*, 1995), and decreased sensitivity to vasorelaxant agents (Lu and Kassab 2011). Whilst morphological changes in endothelial cells and smooth muscle cells were suggested to be minor (Falloon *et al.*, 1995), wires used in wire myography are associated with a small amount of endothelial injury (Dunn *et al.*, 1994). These alterations in sensitivity to pharmacological agents may be associated with arterial damage caused when using wire myography, or may be due to the comparative lack of physiological relevance of wire myography compared to pressure myography. To date only one study has looked at the effects of cannabinoids using pressure myography. Kenny *et al.* (2005) report that, in human myometrial arteries, AEA was unable to cause vasorelaxation. Given that the effects of cannabinoids have not previously been reported in myometrial arteries it is hard to draw any meaningful comparative conclusion from this study. However, to take cannabinoid-induced vasorelaxation research a step closer to the physiological setting, future research should investigate the effects of cannabinoids in perfused arteries.

A further limitation of wire myography is that it relies on the use of pharmacological antagonists and inhibitors, many of which have actions that are beyond their target receptor or enzyme (Pertwee 2005; Fowler 2007). Although controls for this activity were put in place by using different analogues to inhibit the same target site and

by using concentrations that avoid off target actions, this thesis has shown several novel findings that need to be further substantiated using molecular pharmacological techniques or immunochemical techniques. In Chapter 3, the finding that 2-AG appears to be metabolised by COX-1 with subsequent prostanoid receptor activity could be further probed. Firstly ELISA techniques could be used to measure COX activity in explanted human mesenteric artery endothelial and smooth muscle cells after exposure to 2-AG. Using similar explanted cells and ELISA technique it would also be possible to assess prostanoid production. Given rapid hydrolysis of prostaglandin glycerol esters, assessment of their formation may prove difficult. However, other studies have incubated 2-AG with cell lines and characterised prostaglandin glycerol ester production using mass spectrometry. Therefore arterial cells could be explanted and incubated with 2-AG and be assessed in the same way. Alternately it is now possible to purchase synthetic, stable, glycerol esters that could be used in conjunction with a wire myograph to assess their potential vasorelaxant effects in human arteries.

Chapter 4 of this thesis describes CBD induced vasorelaxation through CB_1 dependent pathways. Given that binding and activation of CB_1 by CBD is controversial, expression of CB_1 receptors using RT-PCR and radioligand binding of CBD to CB_1 receptors in explanted endothelial or smooth muscle cells would have added strength to this argument. CBD was also inhibited by capsaicin pre-treatment. Whilst CBD has previously been shown to activate TRPV1 channels in other studies, the present study could have confirmed this in human smooth muscle or endothelial cells using patch clamp techniques similar to those of Zygmunt *et al.* (1999).

Chapter 5 showed that the effects of AEA are not mediated by the TRPV1 receptor. Therefore, despite the findings of CBD, carrying out concentration-response curves to capsaicin would aid in showing the functional role of this receptor in human mesenteric arteries. Also, investigations into optimal conditions for AEA-induced activation of the TRPV1 receptor as outlined in section 6.1.2 would aid in understanding the physiological relevance of AEA's interactions with TRPV1 or lack thereof in the human mesenteric arteries.

Both 2-AG and CBD-induced vasorelaxation was dependent on potassium-mediated hyperpolarisation (this mechanism was not probed

in AEA-induced vasorelaxation). Further characterisation of this could be achieved using explanted smooth muscle or endothelial cells in conjunction with patch clamp techniques or pharmacologically using a range of potassium channel blockers including iberiotoxin, apamin, TRAM 34 and glibenclamide. By doing this it could first be shown what channels were activated and secondly, if the activation was caused directly or via receptor/metabolism mediated pathways.

Finally, the studies conducted in this thesis found that the cannabinoids CBD (chapter 4) and AEA (chapter 5) caused time-dependent vasorelaxation of human mesenteric arteries. However, this effect could not be inhibited using a PPARγ antagonist. The potential reason for this lack of responses could be that the arteries used in this study were not big enough. To further probe this finding similar experiments using a known PPARγ agonist such as pioglitazone could be carried out in the presence or absence of a PPARγ antagonist. This would clarify the potential role for PPARγ in human mesenteric arteries. However, as discussed in section 6.1.2, it might be that CBD and AEA exerted time dependent vasorelaxation through stimulation of the CB_1 receptor. To probe this similar methods as those used in Grimsey *et al.* (2010) could be used. In brief the previous study labelled CB_1 receptors in CB_1 receptor expressing cells and stimulated the receptor with a range of compounds until the receptor could no longer be seen on the cell membrane, membranes were then assessed to see if CB_1 receptors were recycled. Carrying out such an experiment would offer insight into cannabinoid receptor stability and signalling in the vasculature.

The influence of patient characteristics may have had significant affects on the findings of this thesis. The acquisition of mesenteric arteries from patients without prior ill health and lower age would have been preferable, however choice was limited by availability. In future studies, peripheral arteries could be sought from healthy patients undergoing surgery for procedures such as hernia repair, open muscle biopsies, caesarean sections and breast reconstructive surgery. These studies would be able to fully probe the effects of a range of cannabinoids in otherwise healthy patients. In such studies it would also be of benefit to assess the levels of endocannabinoids and receptor expression, this would therefore enhance the understanding of physiological expression and function of the endocannabinoid system.

The studies carried out in this thesis have shown that the effects of cannabinoids might be reduced in patients with cardiovascular disease or cardiovascular disease risk factors. Therefore, to fully understand the effect these conditions have on cannabinoid-induced vasorelaxation future work should specifically address individual disease states, which could be done in different ways. One way would be to take arteries from patients having surgery that was not directed at curing or treating an underlying disease such as previously detailed. Given that some of these patients will naturally have cardiovascular disease or cardiovascular disease risk factors, and other patients will be apparently healthy direct comparisons can be made between a range of cannabinoids and disease states. A further way to investigate the role of disease on the effects of cannabinoids would be to take samples from patients with a known disease risk factor such as bariatric patients. In these patients a common disease risk factor will be present however the extent of co-morbidities will differ. Therefore, correlations can be made between the impact of co-morbidities on selected cannabinoids and vascular function. In both these routes of investigation pharmacological characterisation of cannabinoid function could be carried out and there could also be characterisation of receptor expression, enzyme expression and endocannabinoid plasma or whole blood levels, which would further increase the understanding of the role of the endocannabinoid system in vascular disease.

Finally cannabinoids are being increasingly used as therapeutics and also studies suggest that some cannabinoids such as CBD can be tolerated well in humans (Bergamaschi *et al.,* 2011). Therefore cardiovascular parameters could be monitored in patients currently taking cannabinoid-based medication to see if there was any favourable effect. Furthermore non-psychoactive cannabinoids could be administered either systemically or locally to see if cannabinoids cause vasorelaxation or hypotension *in vivo* in humans. Another potential therapeutic option would be the administration of FAAH inhibitors to patients. Such a treatment may increase the amount of free AEA, which could potentially have a systemic hypotensive effect. A final therapeutic strategy could also be to manipulate the metabolism of 2-AG. As Chapter 3 showed, antagonism of contractile prostaglandin receptors enhanced 2-AG vasorelaxation, therefore administration of contractile

prostanoid receptor antagonists to patients may enhance 2-AG induced vasorelaxation.

6.3. Final conclusion

The primary aim of this project was to screen and characterise the pharmacological effects of cannabinoids in human mesenteric arteries. A secondary aim of this study was then to characterise the effects of he most interesting cannabinoids seen in screening experiments. A third aim was to investigate the possibility of cannabinoids causing time-dependent PPARγ-mediated vasorelaxation in human arteries. A final aim, based on the variability of cannabinoid responses in human mesenteric arteries, became the analysis of cannabinoid responses based on patient characteristics. This study shows for the first time that phytocannabinoids, synthetic cannabinoids and endogenous cannabinoids cause vasorelaxation of human mesenteric arteries. This study also shows that differences exist between human mesenteric and animal arteries in the extent of cannabinoid-induced vasorelaxation and the pathways utilised. The vasorelaxation observed in this study was through the specific cannabinoid receptors (CB_1, CB_e,) endovanilloid receptor (TRPV1) and well-established vasomodulators (COX, NO, ion channel modulation). Therefore, it is likely that, in human mesenteric arteries, the endocannabinoid system plays a role in the modulation of vascular tone, which may be susceptible to decline in disease states.

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